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THROMBOXANE BIOSYNTHESIS AND ITS INHIBITION IN MAN.

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M.B.,Ch.B.

Submitted to Glasgow University for the degree of M.D.

August 1987.

Research undertaken at Vanderbilt University School of  
Medicine, Nashville, Tennessee, USA.

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### GENERAL AIMS

To study the endogenous biosynthesis of thromboxane in man and evaluate the biochemical and functional consequences of selective inhibition of thromboxane formation in an appropriate clinical model.



## CONTENTS

Table of Contents	3
List of Tables and Figures	16
Acknowledgements	28
Declaration	30
Summary	31
Abbreviations	34
<u>CHAPTER 1. INTRODUCTION</u>	37
General Introduction	41
HISTORICAL BACKGROUND	42
PATHWAYS OF ARACHIDONIC ACID METABOLISM:	45
Cyclooxygenase Pathway	47
Lipoxygenase Pathways	49
12-lipoxygenase	51

15-lipoxygenase	51
5-lipoxygenase	52
Epoxygenase Pathway	54
Interactions Between the Pathways Of Arachidonic Acid Metabolism	55
CYCLOOXYGENASE PRODUCTS OF ARACHIDONIC ACID: Their Metabolism and Biological Properties	56
Prostaglandin Endoperoxides:	
Metabolism	56
Biological Properties-	
Cyclic endoperoxides	58
PGD <sub>2</sub> , PGE <sub>2</sub> and PGF <sub>2α</sub>	58
Thromboxane A <sub>2</sub> :	
Metabolism	59
Biological Properties-	
Effects on platelets	61
Effects on smooth muscle	62
Prostacyclin:	
Metabolism	63
Biological Properties-	
Effects on platelets	67
Effects on vascular smooth muscle	68

PLATELET-VESSEL WALL INTERACTIONS: Role of Arachidonic Acid Metabolites	69
Platelet Adhesion	69
Platelet Aggregation	71
Platelet Secretion	73
Prostacyclin-Thromboxane "Balance" in Haemostasis and Thrombosis	74
Importance of Prostacyclin	76
THE ROLE OF THROMBOXANE IN HUMAN DISEASE	79
Syndromes Putatively Associated with Enhanced Thromboxane Synthesis	79
Assessing Thromboxane Synthesis and Platelet Activation: methodological problems	80
APPROACHES TO INHIBITION OF THROMBOXANE FORMATION IN MAN	82
1. Inhibition of Enzymes in the Arachidonic Acid Cascade:	83
Cyclooxygenase-	
Irreversible inhibition	83

Reversible Inhibition	85
Thromboxane Synthase	86
2.Alternative Fatty Acid Substrates	88
SUMMARY AND SPECIFIC OBJECTIVES	91
<u>CHAPTER 2. METHODS</u>	93
I.PROSTAGLANDIN METABOLITES	97
General Considerations	97
<u>Thromboxane:</u>	100
Serum Thromboxane B <sub>2</sub>	102
Urinary 2,3-dinor-thromboxane B <sub>2</sub> (Tx-M)-	
Sample collection	103
Internal standard	104
Methoxime derivatization and extraction	105
Further purification and derivatization	106
Gas chromatography/mass spectrometry	107

Evidence that Tx-M derives predominantly from platelets-	109
Relationship between Tx-M excretion and platelet count	110
Effect of increased platelet turnover on Tx-M	111
Tx-M excretion in patients with platelet activation in vivo	112
Effect of aspirin on Tx-M: pattern of inhibition and recovery	113
Review of other studies	114
Measurement of Urinary Thromboxane B <sub>2</sub>	115
Calculations	116
<u>Prostacyclin</u>	117
Urinary 2,3-dinor-6-keto-PGF <sub>1α</sub> (PGI-M):	
Sample collection	117
Internal standard	117
Derivatization and purification	118
Gas chromatography/mass spectrometry	119
II. PLATELET FUNCTION	121

General Considerations	121
Bleeding Time	122
Platelet Aggregation:	123
Preparation of platelet-rich plasma	124
Aggregometry	124
Platelet aggregation in whole blood	126
Platelet Granule Proteins:	127
$\beta$ -thromboglobulin-	
Sample collection and preparation	129
Radioimmunoassay procedure	129
Platelet Factor 4	130
Circulating Platelet Aggregates	132
 III. ASSAYS OF DRUGS AND THEIR METABOLITES	 133
Aspirin and Salicylate:	133
Sample preparation	134
Analytical procedure	134
Gas chromatography/mass spectrometry	135
 3-lH-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-prop anoic acid (UK 38,485)	 135
 Imidazo(1,5-2)pyridine-5-hexanoic acid (CGS 13080)	 136

IV. STATISTICAL METHODS	137
<u>CHAPTER 3. SELECTIVE INHIBITION OF ENDOGENOUS</u>	
<u>THROMBOXANE FORMATION: Studies in Healthy Subjects</u>	138
Introduction	141
I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE:	146
Study Design	147
Effect on platelet thromboxane formation ex vivo	148
Effect on thromboxane biosynthesis	149
Effect on prostacyclin biosynthesis	150
Effects on platelet function	151
Plasma drug levels	153
Discussion	154
II. CYCLOOXYGENASE INHIBITION BY LOW-DOSE ASPIRIN:	162
Effect on thromboxane synthesis in vivo and ex vivo	163

Effect on prostacyclin biosynthesis	164
Effects on platelet function	165
Discussion	166
III.COMBINED INHIBITION OF PLATELET CYCLOOXYGENASE AND THROMBOXANE SYNTHASE:	172
Effects on thromboxane formation in vivo and ex vivo	173
Effect on prostacyclin biosynthesis	174
Effects on platelet function	175
Discussion	176
IV.INHIBITION OF THROMBOXANE SYNTHESIS IN VIVO AND EX VIVO: Evidence that the Relationship is Non-linear	179
Study Design	180
Results	181
Discussion	183
V.PRESYSTEMIC ACETYLATION OF PLATELET CYCLOOXYGENASE:	185



Design of the Model	187
Effects on platelet thromboxane formation	189
Effects on platelet function	190
Evidence for a presystemic site of action-	
Plasma aspirin and salicylate levels	191
Effect on prostacyclin biosynthesis	192
Recovery of platelet thromboxane synthesis	192
Discussion	193
CONCLUSION	200

#### CHAPTER 4. THROMBOXANE BIOSYNTHESIS IN HUMAN SYNDROMES OF PLATELET ACTIVATION

204

Introduction	207
I. SEVERE ATHEROSCLEROSIS:	210
Study Design-	
Patient selection	211
Analytical methods	213
Evidence for Platelet Activation in vivo	214

Thromboxane Biosynthesis (Tx-M)	215
Discussion	216
 II.SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON:	 219
Study Design-	
Patient selection	220
Analytical methods	221
Evidence for Platelet Activation in vivo	222
Effect of Cooling	224
Discussion	225
 III.MYELOPROLIFERATIVE DISORDERS:	 227
Study Design-	
Patient selection	228
Analytical methods	229
Results	229
Discussion	231
 CONCLUSION	 234

## CHAPTER 5. SELECTIVE INHIBITION OF ENDOGENOUS

### THROMBOXANE FORMATION:

#### Studies in a Human Model of Platelet Activation in vivo 236

Introduction 239

I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE 242

#### Study Design-

Patient selection 244

Protocol 245

Clinical evaluation 247

Analytical methods. 248

#### Results-

Evidence of platelet activation in vivo 249

Effects on thromboxane synthesis in vivo and ex vivo 249

Effects on prostacyclin biosynthesis 251

Effects on platelet function 251

Plasma drug levels 252

Clinical evaluation 253

Discussion 254

II. INHIBITION OF THROMBOXANE BIOSYNTHESIS BY FISH-OIL ADMINISTRATION:	262
Study Design-	
Patient selection	264
Protocol	265
Analytical methods	266
Results-	
Evidence of platelet activation in vivo	268
Effects on thromboxane synthesis in vivo and ex vivo	268
Effects on prostacyclin biosynthesis	270
Effects on platelet function	270
Effects on erythrocyte fatty acids	271
Clinical evaluation	272
Discussion	273
CONCLUSION	277
 <u>CHAPTER 6. FINAL DISCUSSION AND CONCLUSION</u>	 279

APPENDIX I 289

Prostaglandin Metabolites:normal values

Serum thromboxane B<sub>2</sub>

Urinary Tx-M and thromboxane B<sub>2</sub>

Urinary PGI-M

APPENDIX II 291

Platelet Function:normal values

Bleeding time

Platelet aggregation

Platelet granule proteins

Circulating platelet aggregate ratio

APPENDIX III 293

Reagents and Equipment

APPENDIX IV 298

Ethical Considerations

REFERENCES 300

List of Publications 388

## List of Tables and Figures

### Tables

Table 1.1 Syndromes putatively associated with enhanced thromboxane synthesis.	79
Table 2.1 Tx-M excretion in patients with increased platelet turnover.	111
Table 2.2 Tx-M excretion in patients with platelet activation in vivo.	112
Table 3.1 PGI-M excretion after administration of UK 38,485 to healthy subjects.	150
Table 3.2 Platelet aggregation in PRP in response to 1.33mM arachidonic acid after administration of UK 38,485.	151
Table 3.3 Platelet aggregation in PRP in response to 0.66mM arachidonic acid after administration of UK 38,485.	151
Table 3.4 Platelet aggregation in PRP in response to 19 ug/ml collagen after administration of UK 38,485.	152
Table 3.5 Platelet aggregation in PRP in	

response to 1.9 ug/ml collagen after administration  
of UK 38,485. 152

Table 3.6 Platelet aggregation in PRP in  
response to 10uM adrenaline after administration of  
UK 38,485. 152

Table 3.7 Platelet aggregation in PRP in  
response to 5uM adrenaline after administration of  
UK 38,485. 152

Table 3.8 Platelet aggregation in PRP in  
response to 1uM adrenaline after administration of  
UK 38,485. 152

Table 3.9 Platelet aggregation in whole blood in  
response to 0.66mM arachidonic acid after  
administration of UK 38,485. 152

Table 3.10 Platelet aggregation in whole blood  
in response to 9.5 ug/ml collagen after  
administration of UK 38,485. 152

Table 3.11 Plasma concentrations of UK 38,485 in  
healthy subjects after single-dose administration. 153

Table 3.12 Tx-M, serum thromboxane B<sub>2</sub> and  
plasma salicylate after administration of 120mg  
sodium salicylate. 163

Table 3.13 Effects of low-dose aspirin (120mg)  
on platelet aggregation and bleeding time in  
healthy subjects. 166

Table 3.14 Tx-M excretion after combined

cyclooxygenase and thromboxane synthase inhibition. 173

Table 3.15 Serum thromboxane B<sub>2</sub> generation  
after combined cyclooxygenase and thromboxane  
synthase inhibition. 173

Table 3.16 PGI-M excretion after combined  
cyclooxygenase and thromboxane synthase inhibition. 174

Table 3.17 Effects on platelet function of  
combined cyclooxygenase and thromboxane synthase  
inhibition. 175

Table 3.18 Plasma concentrations of UK 38,485  
administered in combination with aspirin or alone. 175

Table 3.19 Urinary excretion of prostaglandin  
metabolites (Tx-M and PGI-M) after administration  
of "slow-release" low-dose aspirin. 190

Table 3.20 Platelet aggregation after  
administration of "slow-release" low-dose aspirin. 191

Table 4.1 Eicosanoid synthesis and platelet  
function in patients with myeloproliferative  
disorders. 230

Table 5.1 Tx-M excretion after administration of  
CGS 13080 to patients with atherosclerosis. 250

Table 5.2 Urinary excretion of Tx-M and  
thromboxane B<sub>2</sub> after chronic administration  
of CGS 13080 to patients with atherosclerosis. 250



Table 5.3 PGI-M excretion after administration of CGS 13080 to patients with atherosclerosis.	251
Table 5.4 Effect of aspirin (325mg) on platelet aggregation, bleeding time and serum thromboxane B <sub>2</sub> in patients with atherosclerosis.	252
Table 5.5 Plasma concentrations of CGS 13080 in patients with atherosclerosis	253
Table 5.6 Effect of fish-oil on indices of platelet function in patients with atherosclerosis.	269
Table 5.7 Incorporation of eicosapentaenoic acid and arachidonic acid into erythrocyte phospholipids in patients with atherosclerosis.	272
APPENDIX I	289
Table AI.1 Serum thromboxane B <sub>2</sub> : normal values.	
APPENDIX II	291
Table AII.1 Platelet aggregation in PRP in response to 1.33mM arachidonic acid: normal values in healthy subjects.	
Table AII.2 Platelet aggregation in PRP in response to 0.66mM arachidonic acid: normal values in healthy subjects.	
Table AII.3 Platelet aggregation in PRP in response to 19 ug/ml collagen: normal values in healthy subjects.	
Table AII.4 Platelet aggregation in PRP in	

response to 1.9 ug/ml collagen: normal values in healthy subjects.

Table AII.5 Platelet aggregation in PRP in response to 10uM adrenaline: normal values in healthy subjects.

Table AII.6 Platelet aggregation in PRP in response to 5uM adrenaline: normal values in healthy subjects.

Table AII.7 Platelet aggregation in PRP in response to 1uM adrenaline: normal values in healthy subjects.

Table AII.8 Platelet aggregation in whole blood in response to 0.66mM arachidonic acid: normal values in healthy subjects.

Table AII.9 Platelet aggregation in whole blood in response to 9.5 ug/ml collagen: normal values in healthy subjects.

### Figures

Figure 1.1 Pathways of arachidonic acid metabolism.

45

Figure 1.2 Cyclooxygenase pathway of arachidonic acid metabolism.

48

Figure 1.3 Lipoxygenase pathways of arachidonic

acid metabolism.	50
Figure 1.4 Epoxygenase pathways of arachidonic acid metabolism.	54
Figure 1.5 Major metabolic pathways for thromboxane B <sub>2</sub>	60
Figure 1.6 Metabolism of prostacyclin in man.	65
Figure 1.7 Vascular and intravascular consequences of platelet activation.	70
Figure 1.8 Vascular factors which modify thrombosis and atherogenesis.	76
Figure 1.9 Inhibition of cyclooxygenase: effects on platelet-vessel wall interactions.	82
Figure 1.10 Inhibition of thromboxane synthase: effects on platelet-vessel wall interactions.	82
Figure 1.11 Alternative fatty acid substrates for prostaglandin synthesis.	89
Figure 2.1 Derivatization of Tx-M: methoxime formation in urine.	105
Figure 2.2 Derivatization of Tx-M: bonding to the phenylboronic acid column.	105
Figure 2.3 Thin-layer chromatogram of authentic standards of Tx-M and PGI-M.	106
Figure 2.4 Negative ion-chemical ionization mass spectrum of the methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivative of Tx-M.	108

Figure 2.5 Selected ion monitoring trace for the methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivative of Tx-M and the tetradeuterated internal standard. 108

Figure 2.6 Relationship between Tx-M and platelet count in healthy subjects. 110

Figure 2.7 Inhibition and recovery of Tx-M after single-dose administration of 650mg aspirin. 113

Figure 2.8 Selected ion monitoring trace of the methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivative of thromboxane B<sub>2</sub> and the tetradeuterated internal standard. 115

Figure 2.9 Selected ion monitoring trace of the methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivative of PGI-M and the tetradeuterated internal standard. 120

Figure 2.10 Calculation of the LT<sub>50</sub> (lag time to achieving 50% of maximal aggregation). 125

Figure 3.1 Chemical formula of 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid (UK 38,485). 146

Figure 3.2 Effect of UK 38,485 on serum thromboxane B<sub>2</sub> in healthy subjects. 148

Figure 3.3 Effect of UK 38,485 on Tx-M excretion in healthy subjects. 148

Figure 3.4 Effect of UK 38,485 on the bleeding time in healthy subjects.	152
Figure 3.5 Effects of low-dose aspirin (120mg) on serum thromboxane $B_2$ generation and Tx-M excretion in healthy subjects.	163
Figure 3.6 Percentage reduction in PGI-M and Tx-M excretion after administration of low-dose aspirin to healthy subjects.	165
Figure 3.7 Relationship between inhibition of serum thromboxane $B_2$ generation ex vivo and Tx-M excretion.	182
Figure 3.8 Presystemic action of low-dose aspirin.	187
Figure 3.9 Serum thromboxane $B_2$ after administration of simulated "slow-release" low-dose aspirin.	189
Figure 3.10 Serum thromboxane $B_2$ after administration of a single 20mg dose of aspirin and repeated administration of 20 lmg doses of aspirin.	189
Figure 3.11 Arachidonate-induced platelet aggregation after administration of 4 lmg doses of aspirin.	191
Figure 3.12 Recovery of serum thromboxane $B_2$ after administration of simulated "slow-release" low-dose aspirin.	192

Figure 4.1 Indices of platelet activation in vivo in patients with severe atherosclerosis.	214
Figure 4.2 PGI-M excretion in patients with severe atherosclerosis and healthy control subjects.	215
Figure 4.3 Tx-M excretion in patients with severe atherosclerosis and healthy control subjects.	215
Figure 4.4 Urinary excretion of Tx-M and PGI-M in patients with systemic sclerosis and Raynaud's phenomenon.	223
Figure 4.5 Effect of cooling on Tx-M excretion in patients with systemic sclerosis and Raynaud's phenomenon.	224
Figure 4.6 Effect of cooling on PGI-M excretion in patients with systemic sclerosis and Raynaud's phenomenon.	224
Figure 4.7 Effect of cooling on plasma concentrations of $\beta$ -thromboglobulin and platelet factor 4 in patients with systemic sclerosis and Raynaud's phenomenon.	224
Figure 5.1 PGI-M excretion after oral administration of various thromboxane synthase inhibitors.	244
Figure 5.2 Excretion of Tx-M and PGI-M after administration of CGS 13080 to patients with	

atherosclerosis.	249
Figure 5.3 Effect of CGS 13080 on serum thromboxane B <sub>2</sub> generation in patients with atherosclerosis.	250
Figure 5.4 Plasma concentrations of β-thromboglobulin and platelet factor 4 after administration of CGS 13080 and placebo to patients with atherosclerosis.	251
Figure 5.5 Effect of CGS 13080 on the circulating platelet aggregate ratio in patients with atherosclerosis.	252
Figure 5.6 Effect of CGS 13080 on the bleeding time in patients with atherosclerosis.	252
Figure 5.7 Effects of CGS 13080 on platelet function in patients with atherosclerosis.	252
Figure 5.8 Platelet aggregation in response to collagen after administration of CGS 13080 to patients with atherosclerosis.	252
Figure 5.9 Relationship between plasma concentrations of CGS 13080 and the effects on indices of thromboxane synthesis and platelet function.	253
Figure 5.10 Effect of chronic administration of CGS 13080 on the ankle:brachial systolic pressure ratio in patients with severe atherosclerotic peripheral vascular disease.	253
Figure 5.11 Selected ion monitoring traces of urinary eicosanoid metabolites before after fish-oil	

administration.

267

Figure 5.12 Urinary excretion of metabolites of thromboxanes and prostacyclins before and during fish-oil ingestion (10g daily) in patients with atherosclerosis.

269

Figure 5.13 Tx-M excretion after administration of a lower (1g) dose of fish oil to two patients with atherosclerosis.

269

Figure 5.14 Effect of fish-oil on the bleeding time in patients with atherosclerosis.

270

Figure 5.15 Effect of fish-oil on plasma  $\beta$ -thromboglobulin in patients with atherosclerosis.

270

#### APPENDIX I

289

Figure AI.1 Relationship between serum thromboxane  $B_2$  and age.

Figure AI.2 Relationship between serum thromboxane  $B_2$  and sex.

Figure AI.3 Relationship between serum thromboxane  $B_2$  and platelet count.

Figure AI.4 Urinary excretion of Tx-M and thromboxane  $B_2$ : normal values in healthy subjects.

Figure AI.5 Urinary excretion of Tx-M: effect of sex.

Figure AI.6 Urinary excretion of Tx-M: intraindividual variation.



Figure AI.7 Effect of age on Tx-M excretion.

Figure AI.8 Urinary excretion of PGI-M: normal values in healthy subjects.

Figure AI.9 Urinary excretion of PGI-M: intraindividual variation.

Figure AI.10 Effect of age on PGI-M excretion.

## APPENDIX II

291

Figure AII.1 Bleeding time: normal values in healthy subjects.

Figure AII.2 Plasma concentrations of  $\beta$ -thromboglobulin and platelet factor 4: normal values in healthy subjects.

Figure AII.3 Relationship between plasma  $\beta$ -thromboglobulin and platelet factor 4: exclusion of samples with artifactually high platelet factor 4 levels.

Figure AII.4 Circulating platelet aggregate ratio: normal values in healthy subjects.

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### Declaration

I hereby declare that this thesis has been composed entirely by myself and that the studies contained herein have principally been conducted solely by myself. Where collaboration with others was involved this is defined and acknowledged in the relevant chapters.

## SUMMARY

The principal product of arachidonic acid in the platelet is thromboxane  $A_2$ . This compound is the most potent endogenous platelet agonist and vasoconstrictor known in man. Thromboxane  $A_2$  has been implicated in diverse human diseases in which platelet activation and vasospasm are known to occur. There has therefore been considerable interest in the development of therapeutic regimens designed to inhibit thromboxane biosynthesis. The studies described in this thesis investigated endogenous formation of thromboxane in human syndromes of platelet activation and examined the biochemical and functional effects of different approaches to inhibition of thromboxane production in health and disease.

Thromboxane biosynthesis was found to be enhanced in patients with evidence of platelet activation in vivo. There was a concomitant increase in endogenous prostacyclin biosynthesis in

such patients. Prostacyclin, the major product of arachidonic acid metabolism in vascular endothelium, is a potent vasodilator and inhibits platelet aggregation in response to all platelet agonists. Evidence from these and other studies supports the hypothesis that this increase in prostacyclin synthesis reflects enhanced platelet-vessel wall interactions in patients with platelet activation in vivo. The importance of prostacyclin as a local regulator of platelet-vascular interactions in this setting provides the rationale for therapeutic regimens which are able to enhance, or at least maintain, prostacyclin formation in these conditions.

Specific inhibitors of the enzyme thromboxane synthase were shown to increase endogenous prostacyclin synthesis coincident with their inhibitory activity on platelet thromboxane generation. In contrast, aspirin, although a potent inhibitor of thromboxane production and platelet function, significantly depressed prostacyclin production even when administered in low doses. In patients with platelet activation in vivo and enhanced biosynthesis of thromboxane, thromboxane production was markedly reduced both by a

thromboxane synthase inhibitor and by dietary supplementation with fish-oil rich in eicosapentaenoic acid. However, complete, cumulative inhibition of thromboxane formation was not achieved and platelet activation persisted.

The results suggested that accumulated endoperoxides substitute in vivo for the proaggregatory effects of thromboxane  $A_2$  when further metabolism of arachidonic acid is blocked by inhibitors of thromboxane synthase. Also, in order to suppress platelet activation in these conditions, inhibition of the platelet capacity to generate thromboxane must be virtually complete and maintained throughout the dosing interval. These studies indicate that to achieve that objective research should be directed towards the development of new, long-acting thromboxane synthase inhibitors, to be used in combination with endoperoxide/thromboxane  $A_2$  receptor antagonists, and also towards further evaluation of long-term administration of pharmacological doses of fish-oil.

### Abbreviations

AA: arachidonic acid

ADP: adenosine diphosphate

ASA: aspirin

B-TG: B-thromboglobulin

CGS 13080: imidazo(1,5-2)pyridine-5-hexanoic acid

CPA ratio: circulating platelet aggregate ratio

DCHA: docosahexanoic acid

EET: epoxy-eicosatrienoic acids

EPA: eicosapentaenoic acid

GC/MS: gas chromatography/mass spectrometry

HETE: hydroxyeicosatetraenoic acid

HPETE/HPAA: hydroperoxyeicosatetraenoic acid

5HT: 5-hydroxytryptamine

LT: leukotriene

LT<sub>50</sub>: lag time to achieving 50% of maximal  
aggregation

MO HCl: methoxyamine hydrochloride

MO-PFB-TMS: methoxime-pentafluorobenzyl-trimethylsilyl

m/z: mass/charge

NA: no aggregation or not available, as stated



ND: not detected

NSAID: non-steroidal anti-inflammatory drugs

PA: primary wave of platelet aggregation

PAF: platelet activating factor

PDGF: platelet-derived growth factor

PF4: platelet factor 4

PGD<sub>2</sub>: prostaglandin D<sub>2</sub>

PGE<sub>1</sub> /E<sub>2</sub>: prostaglandin E<sub>1</sub> /E<sub>2</sub>

PGF<sub>1 $\alpha$</sub>  /F<sub>2 $\alpha$</sub> : prostaglandin F<sub>1 $\alpha$</sub>  /F<sub>2 $\alpha$</sub>

PGG<sub>2</sub>: prostaglandin G<sub>2</sub>

PGH<sub>2</sub>: prostaglandin H<sub>2</sub>

PGI<sub>2</sub>: prostacyclin

PGI<sub>3</sub>: prostaglandin I<sub>3</sub>

PGI-M or PGI<sub>2</sub>-M: 2,3-dinor-6-keto-prostaglandin

F<sub>1 $\alpha$</sub>

PGI<sub>3</sub>-M: 2,3-dinor-6-keto-17-ene-prostaglandin

F<sub>1 $\alpha$</sub>

PRP: platelet-rich plasma

SA: secondary wave of platelet aggregation

SEM: standard error of the mean

Tmax<sub>6</sub>: height of the platelet aggregation trace 6  
minutes after addition of the agonist

TxB<sub>2</sub> /A<sub>2</sub>: thromboxane B<sub>2</sub> /A<sub>2</sub>

Tx-M or TxB<sub>2</sub>-M: 2,3-dinor-thromboxane B<sub>2</sub>

TxB<sub>3</sub>-M: 2,3-dinor-17-ene-thromboxane B<sub>2</sub>

UK 38,485:

3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-  
propanoic acid

## Chapter 1

### INTRODUCTION

## General Introduction

### HISTORICAL BACKGROUND

### PATHWAYS OF ARACHIDONIC ACID METABOLISM:

#### Cyclooxygenase Pathway

#### Lipoxygenase Pathways-

12-lipoxygenase

15-lipoxygenase

5-lipoxygenase

#### Epoxygenase Pathway

### Interactions Between the Pathways of Arachidonic Acid Metabolism

### CYCLOOXYGENASE PRODUCTS OF ARACHIDONIC ACID:

### Their Metabolism and Biological Properties

#### Prostaglandin Endoperoxides:

Metabolism

Biological Properties-

Cyclic endoperoxides

PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>

Thromboxane A<sub>2</sub>:

Metabolism

Biological Properties-

Effects on platelets

Effects on smooth muscle

Prostacyclin:

Metabolism

Biological Properties-

Effect on platelets

Effect on vascular smooth muscle

PLATELET-VESSEL WALL INTERACTIONS: Role of  
Arachidonic Acid Metabolites

Platelet Adhesion

Platelet Aggregation

Platelet Secretion

Prostacyclin-Thromboxane "Balance" in  
Haemostasis and Thrombosis

Importance of Prostacyclin

THE ROLE OF THROMBOXANE IN HUMAN DISEASE

Syndromes Putatively Associated with Enhanced  
Thromboxane Synthesis

Assessing Thromboxane Synthesis and Platelet  
Activation: methodological problems

APPROACHES TO INHIBITION OF THROMBOXANE  
FORMATION IN MAN

1. Inhibition of Enzymes in the Arachidonic Acid  
Cascade:

Cyclooxygenase-

Irreversible Inhibition

Reversible Inhibition

Thromboxane Synthase

2. Alternative Fatty Acid Substrates

SUMMARY AND SPECIFIC OBJECTIVES

## General Introduction

Thromboxane  $A_2$  is the major metabolite of arachidonic acid formed in the platelet. It is the most potent endogenous platelet agonist and vasoconstrictor known in man and has therefore been implicated in the pathogenesis of acute thrombotic events and in the evolution of diffuse vasoocclusive disease. The biological properties of thromboxane  $A_2$  provide a rationale for the development of regimens to prevent or reduce thromboxane formation in such conditions. These studies aim to investigate the pharmacology of selective inhibitors of thromboxane synthesis in man. Furthermore, in order to identify potential therapeutic targets and the likely impact of such an approach in a clinical setting, human models of platelet activation were sought in which thromboxane biosynthesis was increased.

## HISTORICAL BACKGROUND

The important role of platelets in normal haemostasis and the development of thrombosis was first recognized by Juilius Bizzozero in a monograph published in 1882 (1). He showed that platelets rapidly accumulated at sites of vascular injury to form a haemostatic plug and made the important observation that, in contrast to damaged vessels, normal undamaged endothelium appeared to present a non-thrombogenic surface (2). Although considerable progress has been made since that time in elucidating the biochemical basis for the interaction between platelets and vascular endothelium, the mechanisms underlying this complex process remain poorly understood and the role of arachidonic acid metabolites has only been appreciated in the last two decades.

Arachidonic acid is the principal precursor of a group of compounds with a wide variety of potent biological effects, the prostaglandins. Von Euler first proposed the name "prostaglandin" in 1935 to describe an endogenous



substance in human semen with vasodepressor and smooth muscle stimulating activity (3). Thirty years later Kloeze showed that one of the prostaglandins, prostaglandin E, inhibited ADP-induced platelet aggregation (4). This was followed by Vane's observation in 1971 that non-steroidal anti-inflammatory agents caused a dose-dependent reduction in prostaglandin synthesis in guinea pig lung homogenates, leading him to propose inhibition of prostaglandin formation as the biochemical mechanism of action for aspirin-like drugs (5), which had long been known to profoundly inhibit platelet aggregation. At the same time Smith and Willis confirmed that aspirin inhibited prostaglandin production in human platelets (6).

A series of important papers was subsequently published by Samuelsson's group at the Karolinska Institutet in Stockholm and by Vane and his colleagues at the Wellcome Research Laboratories in Kent describing the metabolism of arachidonic acid to the potent eicosanoids, thromboxane  $A_2$  and prostacyclin ( $PGI_2$ ), in platelets and vascular tissue respectively (7-12). Samuelsson's group identified thromboxane  $A_2$  (8) as a highly unstable intermediate formed from prostaglandin endoperoxides which caused irreversible platelet aggregation and which constituted the major component of rabbit aorta contracting substance (RCS), described some years

previously by Piper and Vane (13). Thromboxane A<sub>2</sub> has recently been chemically synthesized and the bicyclic oxetane structure originally suggested by Hamberg et al confirmed by workers at Columbia University and the Upjohn Company (14). Moncada et al found that prostaglandin endoperoxides could also be transformed by an enzyme isolated from rabbit or pig aortae to an unstable substance which inhibited platelet aggregation and relaxed isolated blood vessels (10). Initially known as PGX (11), its chemical structure was subsequently elucidated and it was renamed prostacyclin (12).

The biological properties of thromboxane A<sub>2</sub> as a potent platelet agonist and vasoconstrictor, and of prostacyclin, the most active endogenous inhibitor of platelet aggregation known and a vasodilator, have focussed interest on their role in the regulation of platelet-vessel wall interactions in man, both under physiological conditions and in the setting of vaso-occlusive disease (15)(16)(17). Consequently, since the first description of these potent eicosanoids almost ten years ago, research has been directed towards methods of inhibiting thromboxane synthesis and/or enhancing prostacyclin formation in disease states in which platelet activation and vasoconstriction are thought to be important mediators.

## PATHWAYS OF ARACHIDONIC ACID METABOLISM

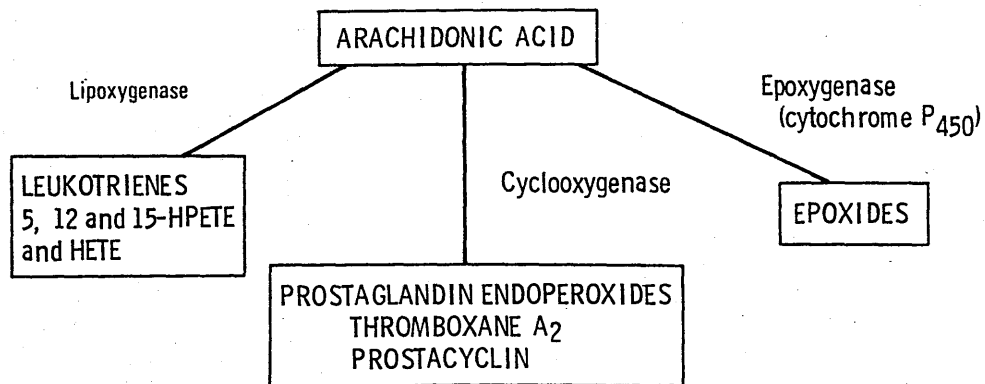
Arachidonic acid, or eicosatetraenoic acid, is the major precursor of prostaglandins in man. It is a 20-carbon polyunsaturated fatty acid and is found within the phospholipids of the cell membrane of all mammalian tissues. After being released from membrane phospholipids, arachidonic acid is rapidly metabolized into oxygenated products via three distinct pathways (Figure 1.1):

(i) the cyclooxygenase pathway, to produce prostanoids and thromboxanes;

(ii) via a series of lipoxygenases, to produce mono- and di- hydroxyeicosatetraenoic acids (HETES) and the leukotrienes; and

(iii) via cytochrome p-450 to form epoxy-eicosatrienoic acids (EETs)(18).

FIGURE 1.1 PATHWAYS OF ARACHIDONIC ACID METABOLISM



Thromboxane  $A_2$ , the prostaglandins, leukotrienes and the epoxygenase products are collectively known as eicosanoids, reflecting their common origin from eicosatetraenoic (arachidonic) acid. In platelets and endothelial cells arachidonic acid metabolism is mainly directed towards the cyclooxygenase pathway although, as outlined below, the potential importance of lipoxygenase products is being increasingly recognized. As recent evidence suggests that leucocytes also play a significant role in platelet-vascular interactions, the following section includes discussion of arachidonic acid metabolism in these cells, in which, by contrast with platelets and endothelial cells, the lipoxygenase pathway predominates. A third pathway of arachidonic acid metabolism, the epoxygenase pathway, has recently been identified in isolated cell suspensions from rabbit renal tissue (19)(20). Whether these compounds are synthesized in man and their possible role remains to be established. In addition, there are now many examples of biochemical interactions between the different cell types as a result of sharing of various precursors and intermediates of arachidonate metabolism.

### Cyclooxygenase Pathway:

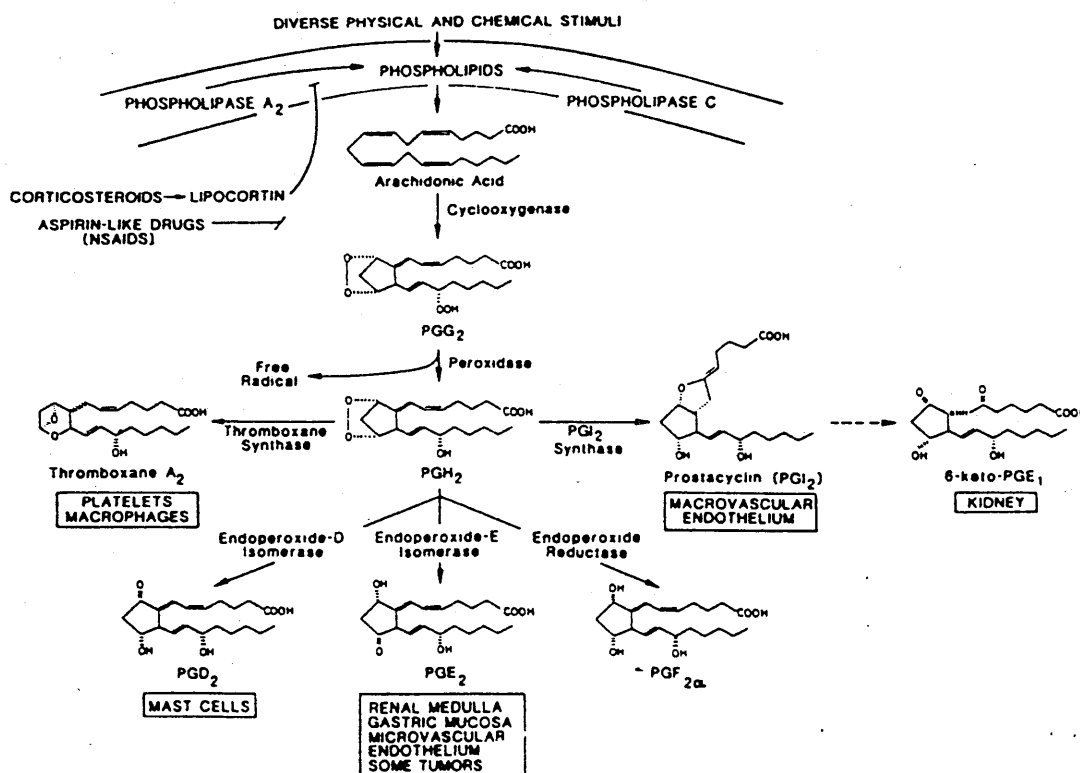
Arachidonic acid is converted into the cyclic endoperoxides, prostaglandin  $G_2$  ( $PGG_2$ ) and prostaglandin  $H_2$  ( $PGH_2$ ) via the action of the cyclooxygenase enzyme. Cyclooxygenase is present in most tissues, with relatively high concentrations in platelets and endothelial cells (7)(21)(22), as well as in the kidney, spleen and lung (23)(24)(25). The enzyme is membrane-associated and has been localized by immunocytochemical studies to the endoplasmic reticulum and nuclear membrane (26).

Cyclooxygenase has now been solubilized and purified to homogeneity (27)(28). It is composed of two subunits with a molecular weight of 72,000 daltons (29). The enzyme catalyses two reactions: the cyclooxygenase reaction in which two molecules of oxygen are incorporated into arachidonate to produce  $PGG_2$ , and the hydroperoxidase reaction in which the 15-hydroperoxide of  $PGG_2$  is converted to a hydroxyl group to produce  $PGH_2$  (Figure 1.2). The cyclooxygenase and peroxidase activities have been shown to reside in a single membrane-associated protein (28). Several investigators

have demonstrated self-deactivation of cyclooxygenase in intact cells suggesting that such a mechanism may be important in regulating prostaglandin synthesis in vivo (30)(31).

The cyclic endoperoxides formed by cyclooxygenase are unstable and are therefore rapidly metabolized to a number of different biologically active products depending on cell type (Figure 1.2). In platelets and macrophages, for example, the principal metabolite is thromboxane  $A_2$  (9)(32)(33), whereas in vascular endothelium it is prostacyclin (11) and in the kidney  $PGE_2$  predominates (34).

FIGURE 1.2 CYCLOOXYGENASE PATHWAY OF ARACHIDONIC ACID METABOLISM.



### Lipoxygenase Pathway:

A series of lipoxygenases has been described in various mammalian tissues which peroxidize arachidonic acid at different carbon atoms. These include:

(i) the 12-lipoxygenase enzyme in platelets (7) and the kidney (35), which converts arachidonate into 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which in turn is rapidly reduced to the corresponding hydroxy acid 12-hydroxyeicosatetraenoic acid (12-HETE);

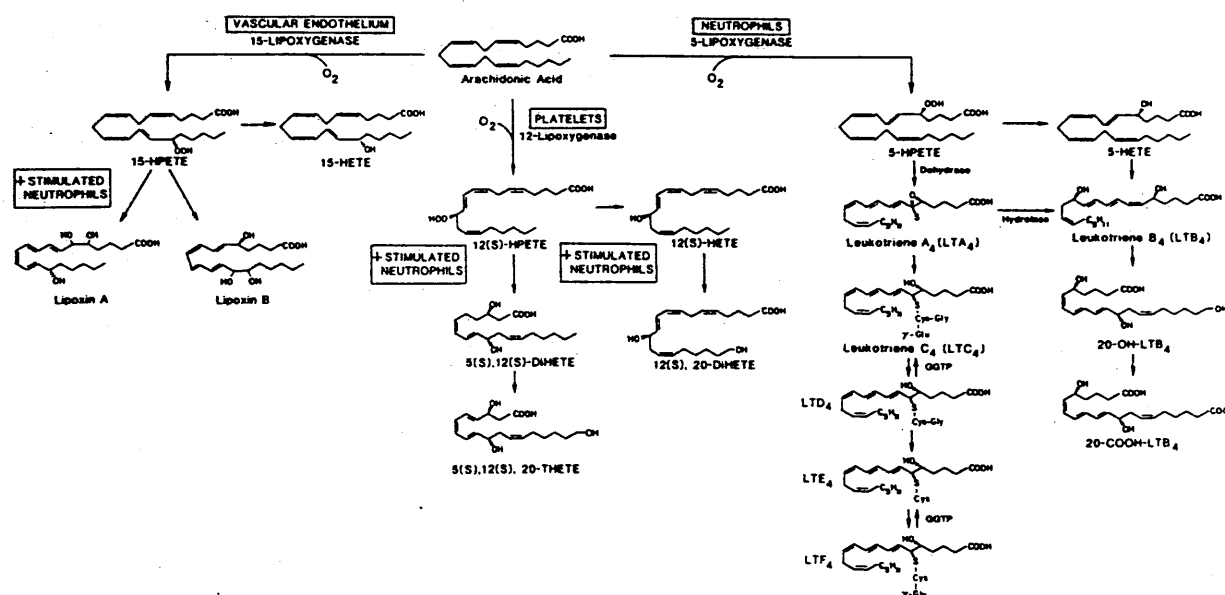
(ii) the 15-lipoxygenase enzyme in endothelial and vascular smooth muscle cells (36), renal glomeruli (35), leucocytes and reticulocytes (37), which reacts with arachidonate to produce 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and 15-HETE; and

(iii) the 5-lipoxygenase enzyme in leucocytes including



neutrophils (38), eosinophils (38) and monocytes (39), which catalyses the conversion of arachidonate to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This in turn may be reduced to the hydroxy acid 5-HETE or further metabolized to generate the leukotrienes (Figure 1.3).

FIGURE 1.3 LIPOXYGENASE PATHWAYS OF ARACHIDONIC ACID METABOLISM.



### 12-lipoxygenase:

The precise biological role of the 12-lipoxygenase products formed by platelets is unclear. Recent reports have shown that in vitro 12-HETE induces migration of aortic smooth muscle cells at very low concentrations (40)(41), leading to the suggestion that this may be of some importance in the pathogenesis of atherosclerosis. 12-HETE is also the major product formed from endogenous arachidonic acid in isolated rat pancreatic islet cells and may be involved in glucose-induced insulin secretion by these cells (42). 12-lipoxygenase products have been implicated in the regulation of cell to cell interactions. Platelet-derived 12-HETE can be utilized by neutrophils to form another eicosanoid, 12,20-diHETE, the function of which is unknown (43). It has also been suggested that 12-HPETE inactivates prostacyclin synthase or may play a role in endothelial cell-dependent relaxation of vascular smooth muscle, although the evidence in support of these hypotheses is inconclusive (44).

### 15-lipoxygenase:

The physiological significance of the 15-lipoxygenase products is also unclear. They have been shown to inhibit prostacyclin synthase (45), platelet aggregation (46) and

leukotriene formation in vitro (47). In atherosclerotic rabbit aortae, synthesis of 15-HETE was found to occur in the absence of any 15-HETE production by normal control aortae, leading to the suggestion that this may be important in explaining why atherosclerotic vessels are prone to thrombosis (48). However, cultured human umbilical vein endothelial cells have been shown to synthesize significant amounts of 15-HETE (36). This observation and the presence of 11-,12- and 15-lipoxygenase products in vascular smooth muscle cells suggest that the products may be involved in platelet-endothelial and neutrophil-endothelial interactions (49)(50). Recent studies in which 15-HPETE was added to stimulated leucocytes have led to the identification of a new series of biologically active compounds, the lipoxins (51). One of these, lipoxin A has been shown to be a potent stimulator of neutrophil superoxide generation and lysosomal elastase release (52).

#### 5-lipoxygenase:

Metabolism of arachidonic acid via the 5-lipoxygenase pathway in leucocytes results in leukotriene formation. The individual leukotrienes possess distinct biological properties and although previously thought to be mainly

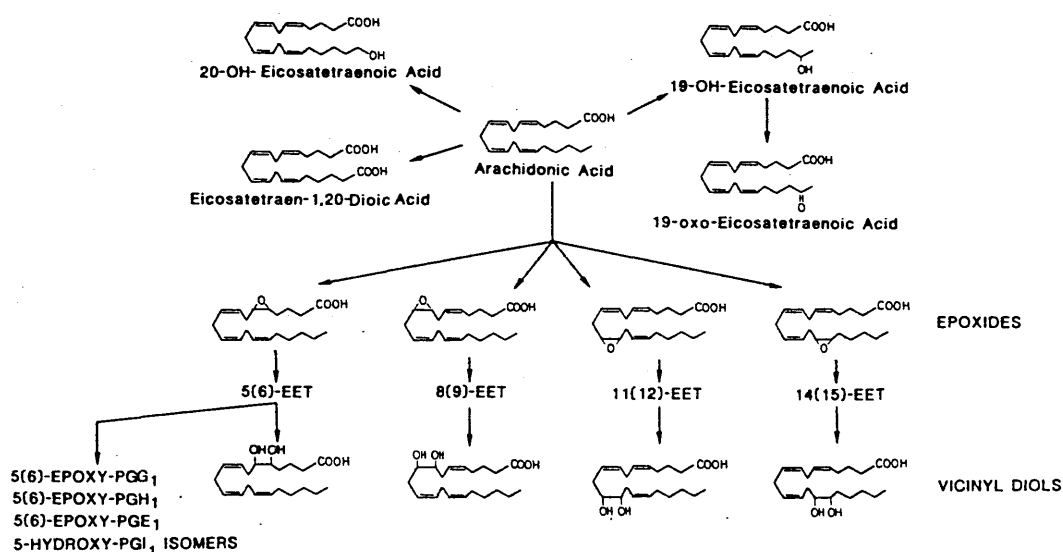
active in acute inflammation (53), their possible role in platelet vessel wall interactions is now being recognized. Leukotriene  $B_4$  is a potent stimulator of neutrophil chemotaxis, chemokinesis, aggregation and adhesion to endothelial cells (54)(55). Of considerable interest is the recent evidence demonstrating that in vitro neutrophils are also able to utilize both platelet-derived arachidonic acid (56) and erythrocyte-derived leukotriene  $A_4$  for leukotriene  $B_4$  synthesis (57).

Leukotriene  $A_4$  may be further converted to its glutathione conjugate, leukotriene  $C_4$ , which is then metabolized to leukotrienes  $D_4$  and  $E_4$ . Leukotrienes  $C_4$  and  $D_4$ , which have been identified as the active constituents of SRS-A (slow-reacting substance of anaphylaxis), contract vascular and bronchial smooth muscle, increase vascular permeability and stimulate mucus secretion (53). Some of the effects of leukotriene  $C_4$  on bronchial smooth muscle are blocked by cyclooxygenase inhibitors, suggesting that thromboxane  $A_2$  or other bronchoconstrictor prostaglandins may contribute to the biological activity of these leukotrienes (58). Leukotriene  $C_4$  has also recently been shown to stimulate prostacyclin synthesis in human endothelial cells (59)(60).

### Epoxygenase Pathway:

Cytochrome p-450 enzymes in microsomal preparations from rabbit renal cortex and liver convert arachidonic acid into a series of epoxides (Figure 1.4), some of which have been shown to have interesting biological properties (19)(20). Two of these compounds, 8,9-EET and 14,15-EET, inhibit human platelet cyclooxygenase (61). In addition, all the EET isomers so far investigated inhibit platelet aggregation, although in some cases this appears to occur by a non-specific mechanism independent of inhibition of thromboxane formation (61).

FIGURE 1.4 EPOXYGENASE PATHWAYS OF ARACHIDONIC ACID METABOLISM



## Interactions between the Pathways of Arachidonic Acid

### Metabolism

Experiments in vitro have provided many examples of biochemical interactions between platelets, leucocytes and endothelial cells in which arachidonic acid precursors or intermediates synthesized by one cell type may be utilized by another. Thus, platelet-derived arachidonate may be metabolized by neutrophils to leukotriene B<sub>4</sub> (56) and 12-HETE from platelets can be utilized by neutrophils to form 12,20-diHETE (43). Other examples are the synthesis by neutrophils of leukotriene B<sub>4</sub> from erythrocyte-derived leukotriene A<sub>4</sub> (57) and of the lipoxins from 15-HPETE (51). In addition, synthesis of prostacyclin by endothelial cells from platelet-derived endoperoxides has been demonstrated by several workers in vitro (62)(63). The observation from human studies that prostacyclin biosynthesis is increased after administration of a thromboxane synthase inhibitor provides evidence that such interactions may also be relevant in vivo (64)(65).

## CYCLOOXYGENASE PRODUCTS OF ARACHIDONIC ACID:

### Their Metabolism and Biological Properties

#### Prostaglandin Endoperoxides

##### Metabolism:

The cyclic endoperoxides,  $\text{PGG}_2$  and  $\text{PGH}_2$ , are unstable prostanoids with a half-life of around 5 minutes (66). They are further metabolized along a number of different pathways depending upon the type of tissue or species studied (Figure 1.2). In human platelets the major product formed from prostaglandin endoperoxides is thromboxane  $\text{A}_2$  by the action of the enzyme thromboxane synthase (9). Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ), prostaglandin  $\text{D}_2$  ( $\text{PGD}_2$ ) and small amounts of prostaglandin  $\text{F}_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ) can also be synthesized by platelets either non-enzymatically or via the action of isomerases (32)(67). The metabolite malondialdehyde (MDA) is produced in approximately equimolar amounts with thromboxane  $\text{A}_2$  (15).

In cultured endothelial cells from large blood vessels endoperoxides are predominantly converted into prostacyclin (68)(69). In the microvasculature, however, recent evidence has shown that significant PGH-PGE isomerase activity is present and that, in this setting,  $\text{PGE}_2$  may be the major product generated from arachidonic acid (70)(71).

Thromboxane synthesis has also been demonstrated in macrophages (33)(72), vascular tissue (73), and the lung (25), kidney (34) and spleen (26). Although human macrophages mainly metabolize arachidonic acid via the 5-lipoxygenase enzyme, they also synthesize significant amounts of thromboxane and  $\text{PGE}_2$  (53)(74). In the kidney the spectrum of arachidonic acid metabolites produced varies in the different parts of the nephron (34)(75). The arteries and arterioles mainly synthesize prostacyclin and  $\text{PGE}_2$  (76). Renal glomeruli produce predominantly prostacyclin, but less  $\text{PGE}_2$ , thromboxane  $\text{B}_2$  and  $\text{PGF}_{2\alpha}$  (77)(78). In the renal medulla the major metabolite synthesized by the collecting tubules and interstitial cells is  $\text{PGE}_2$  (79)(80).



## Biological Properties:

### Cyclic Endoperoxides:

Prostaglandin endoperoxides contract vascular, bronchial and gastrointestinal smooth muscle in vitro (9)(81)(82)(83). Although shown\* to be considerably more potent than  $\text{PGE}_2$  in inducing contraction of rabbit aorta (66), the endoperoxides are approximately 50-fold less potent in this respect than thromboxane  $\text{A}_2$  (9). The effect of  $\text{PGG}_2$  and  $\text{PGH}_2$  on platelets is proaggregatory,  $\text{PGG}_2$  being three times more active than  $\text{PGH}_2$  (66).

### Prostaglandins $\text{D}_2$ , $\text{E}_2$ and $\text{F}_{2\alpha}$ :

$\text{PGD}_2$  and its  $9\alpha, 11\beta$ - $\text{PGF}_{2\alpha}$  metabolite inhibit platelet aggregation (84)(85) by stimulating adenylate cyclase and increasing the intraplatelet concentration of cyclic AMP (86). The potency of  $\text{PGD}_2$  is about 10-20 fold less than prostacyclin (87). The  $9\alpha, 11\beta$ - $\text{PGF}_{2\alpha}$  metabolite also contracts human bronchial smooth muscle (88) and coronary artery rings (89).  $\text{PGE}_2$  is very weakly anti-aggregatory but in low concentrations has been shown to enhance the secondary wave of ADP-induced aggregation (90).  $\text{PGE}_2$  is also a vasodilator and may play a role in the maintenance of renal blood flow and the

glomerular filtration rate under conditions of increased vasoconstrictor activity in the kidney (79).

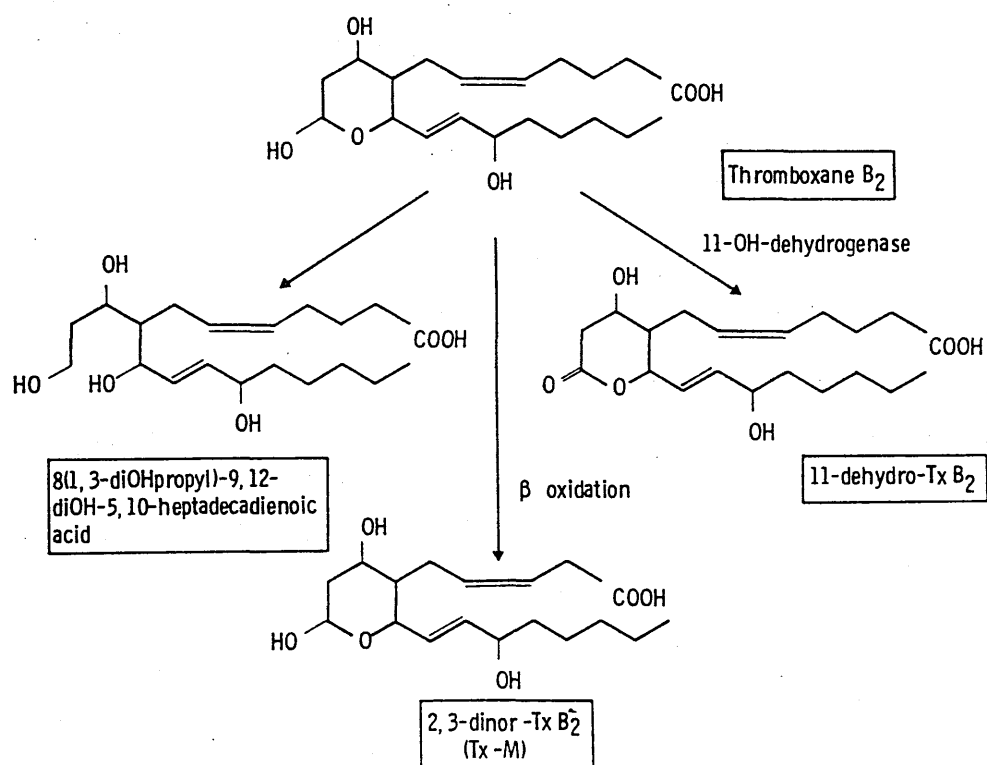
### Thromboxane A<sub>2</sub>

#### Metabolism:

Thromboxane A<sub>2</sub> is a highly unstable intermediate formed from PGH<sub>2</sub> by the action of thromboxane synthase. It has a half-life of approximately 30 seconds (8) and is rapidly hydrolysed non-enzymatically to its stable derivative, thromboxane B<sub>2</sub>. The metabolism of thromboxane B<sub>2</sub> in man (Figure 1.5) has been studied by infusing a supraphysiological dose (6.4 micrograms/minute) intravenously in a human volunteer and identifying the urinary metabolites by gas chromatography/mass spectrometry (91). Using this technique, the major urinary metabolites were shown to be 2,3-dinor-thromboxane B<sub>2</sub> and 11-dehydro-thromboxane B<sub>2</sub>, accounting for 23% and 8% respectively of the total infused dose (91). Although subsequent infusion studies employing more "physiological" doses of thromboxane B<sub>2</sub> (0.1-5 ng/kg/minute) found that only

5.3% of the infused thromboxane  $B_2$  was excreted as the dinor metabolite, this, and 11-dehydro-thromboxane  $B_2$  remain the principal metabolites under normal conditions (92). The discrepancy between these two studies is likely to be due to saturation at the much higher infusion rate of the pathway converting thromboxane  $B_2$  into 11-dehydro-thromboxane  $B_2$  metabolites (93).

FIGURE 1.5 MAJOR METABOLIC PATHWAYS FOR THROMBOXANE  $B_2$



### Biological Properties:

Thromboxane  $A_2$  has two principal biological actions. It is the most potent endogenous inducer of platelet aggregation yet discovered in man (8)(14) and it contracts vascular and bronchial smooth muscle (9)(14)(94)(95).

#### Effects on platelets:

The exact mechanism by which thromboxane activates platelets has not been elucidated. Although there are some reports suggesting that the effects of thromboxane are mediated only indirectly via released ADP (96)(97), some of these were confounded by methodological problems (98) and there is now good evidence that its effects are mediated through thromboxane  $A_2$ /endoperoxide receptor occupancy (99). It is thought that after binding to the receptor, thromboxane raises the intracellular concentration of unbound calcium leading to platelet shape change, primary aggregation and release (100). Experiments involving analogues of thromboxane and the prostaglandin endoperoxides are consistent with a single shared thromboxane  $A_2$ /endoperoxide receptor on the platelet membrane (101)(102)(103). Thromboxane also causes the secondary wave of platelet aggregation initiated by other

agonists such as ADP and adrenaline (104), perhaps by potentiating the exposure of fibrinogen binding sites by these agonists (105). However, ADP does not appear to be essential for the maintenance of thromboxane-induced platelet aggregation (99)(105)(106).

#### Effects on smooth muscle:

Thromboxane  $A_2$  is a potent constrictor of vascular and respiratory smooth muscle (9)(14)(94)(95). Studies in which thromboxane  $A_2$  was generated by incubating  $PGH_2$  with platelet microsomes showed that as little as 30 picomoles of thromboxane  $A_2$  caused marked contraction of rabbit aorta ex vivo (107). There is now good evidence from studies with a variety of thromboxane  $A_2$  and endoperoxide analogues for the existence of different thromboxane  $A_2$ /endoperoxide receptors in platelets from those found in vascular smooth muscle (103)(108).

## Prostacyclin

### Metabolism:

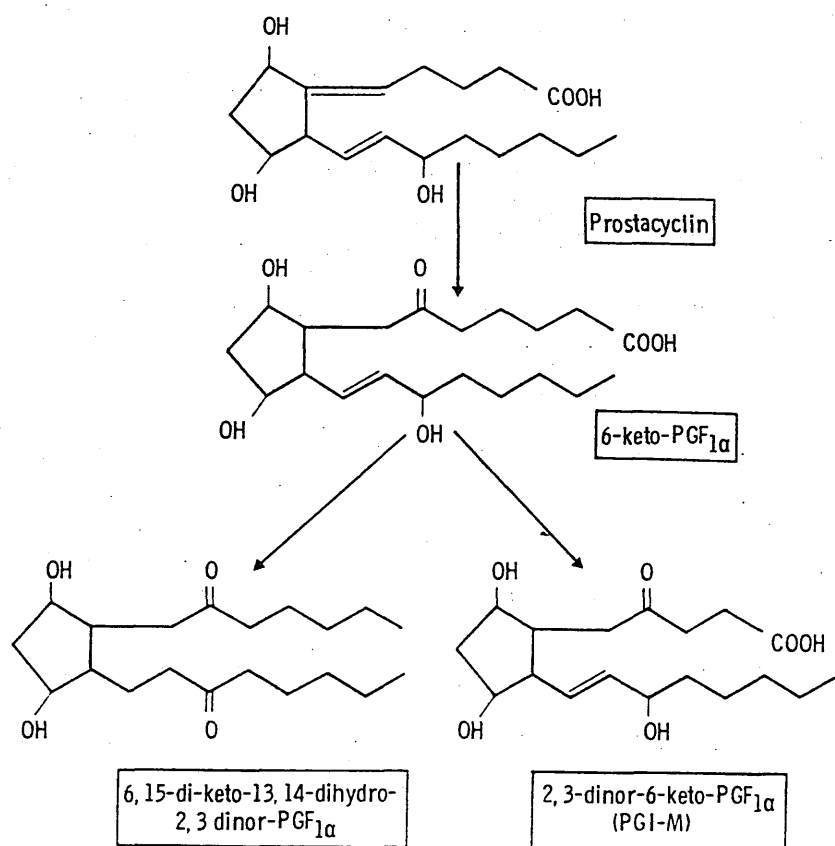
The conversion of  $\text{PGH}_2$  into prostacyclin is catalysed by the enzyme prostacyclin synthase, which is found in the vascular tissues of all mammalian species (15). Prostacyclin synthase has been purified by immunoaffinity chromatography and found to have a molecular weight of 50,000 daltons (109). Early studies showed that in large vessels the endothelium was the major source of prostacyclin synthesis and that vascular smooth muscle had minimal prostacyclin synthase activity (110). Since that time, however, several investigators have demonstrated the capacity of arterial smooth muscle cells to synthesize prostacyclin in vitro (73)(111)(112). Recent experiments using improved immunocytochemical methods indicate that the concentration of prostacyclin synthase is actually the same in different layers of the vasculature, but that considerably lower concentrations of cyclooxygenase are found in arterial smooth muscle compared to endothelial cells (22). It is not clear whether this observation is of functional importance in vivo, although it is possible that reduced cyclooxygenase

activity in vascular smooth muscle may lead to reduced prostacyclin generation at sites of vascular injury.

Prostacyclin synthesis is known to be stimulated in vitro by endogenous platelet agonists such as thrombin (113), ADP (114) and adrenaline (115). Platelet granule constituents, including platelet-derived growth factor (116), platelet activating factor (117) and 5-hydroxytryptamine (118) which are released by activated platelets are also potent stimuli for prostacyclin formation in vitro, as are leukotrienes  $C_4$  and  $D_4$  and  $\alpha$ -interferon (59)(60)(119). Although early in vitro studies suggested that vasoactive mediators, whether endogenous such as adenosine and endothelial cell-derived growth factor (120)(121), or exogenous such as organic nitrates (122), acted via prostacyclin release, more recent work has not confirmed these findings (123)(124)(125)(126). Prostacyclin formation has been shown to be inhibited by 15-lipoxygenase products (45). In addition, prostacyclin synthesis by rabbit aortae ex vivo and by cultured endothelial cells causes deactivation of cyclooxygenase (30)(31). This phenomenon results in a markedly reduced endothelial capacity for continued prostacyclin generation for 24-48 hours (31) and may be important in the regulation of prostacyclin biosynthesis in vivo.

Prostacyclin is unstable, with a half-life of 2-3 minutes in whole blood (127). It is rapidly hydrolysed non-enzymatically to its stable metabolite, 6-keto-PGF<sub>1α</sub> which can be measured in blood or urine (128)(129)(130). The major urinary metabolites, however, are 2,3-dinor-6-keto-PGF<sub>1α</sub> and 6,15-diketo-13,14-dihydro-2,3-dinor-6-keto-PGF<sub>1α</sub> (129)(131) (Figure 1.6).

FIGURE 1.6 METABOLISM OF PROSTACYCLIN IN MAN





Measurement of these prostacyclin metabolites in urine provides a useful non-invasive index of endogenous prostacyclin production in man (131)(132). Employing this approach, the estimated rate of secretion of prostacyclin into the circulation in normal individuals under basal conditions has been shown to be very low- in the order of 0.08-0.10ng/kg/minute (132)- suggesting that, contrary to previous hypotheses (133), it is not a circulating hormone in man.

An alternative pathway of metabolism has also been described in which prostacyclin or 6-keto-PGF<sub>1 $\alpha$</sub>  are converted into 6-keto-PGE<sub>1</sub> via the enzyme 9-hydroxyprostaglandin dehydrogenase (134). The enzyme has been demonstrated in a perfused rabbit liver preparation (135), in human platelets (134) and in human kidney (136). More recently, Pieroni et al clearly showed that the isolated perfused rabbit kidney generates material indistinguishable from 6-keto-PGE<sub>1</sub> by immunoreactive and biological criteria and verified its structure (137). It has been suggested that such a pathway may be important in vivo as 6-keto-PGE<sub>1</sub> has similar biological properties to prostacyclin (137)(138). This remains speculative, however, particularly as studies of the metabolic disposition of prostacyclin in man found no

evidence of this metabolite in urine under physiological conditions (131) or after prostacyclin infusion (129). It therefore seems likely that endogenous 6-keto-PGE<sub>1</sub> production is minimal under normal conditions in humans.

#### Biological Properties:

The two principal biological properties of prostacyclin are inhibition of platelet aggregation and relaxation of vascular smooth muscle. Prostacyclin also appears to exert a "cytoprotective" effect distinct from its anti-platelet and vasodilator activity, for example in preventing endotoxin-induced injury in animal models of shock and reducing myocardial infarct size (139). In addition, studies of cholesteryl ester hydrolytic activity in cultured aortic smooth muscle cells suggest that prostacyclin may be an important regulator of cholesteryl ester metabolism in human arteries (140).

#### Effects on Platelets:

Prostacyclin is the most potent endogenous inhibitor of platelet aggregation yet discovered. It inhibits the aggregation of platelets by all recognized agonists and disaggregates previously aggregated platelets (15). It

exerts these effects by increasing the intra-platelet concentration of cyclic AMP (141). It binds to a common prostacyclin/PGE<sub>1</sub> receptor on the platelet membrane (142), thereby activating adenylate cyclase which in turn leads to accumulation of cyclic AMP (141). The ability of cyclic AMP to prevent platelet aggregation is thought to be mediated by inhibition of the mobilization of fibrinogen and von Willebrand factor binding sites on the platelet membrane (143)(144) and/or a reduction in the intraplatelet concentration of calcium (145)(146). The effects of prostacyclin on platelet adhesion are unclear. Some reports suggest that it possesses inhibitory activity (147)(148)(149), while others have failed to confirm these findings (150).

#### Effects on Vascular Smooth Muscle:

Prostacyclin relaxes vascular strips in vitro in all species examined including man, with the exception of porcine coronary artery and some preparations of human saphenous vein and rat venous tissue (10)(151)(152)(153). It is 4-8 times more potent than PGE<sub>2</sub> and at least 100 times more active than its stable metabolite 6-keto-PGF<sub>1α</sub> (154). Prostacyclin has also been shown to produce vasodilatation and lower diastolic blood pressure when infused into animals (154) and man (155).

PLATELET-VESSEL WALL INTERACTIONS:  
ROLE OF ARACHIDONIC ACID METABOLITES

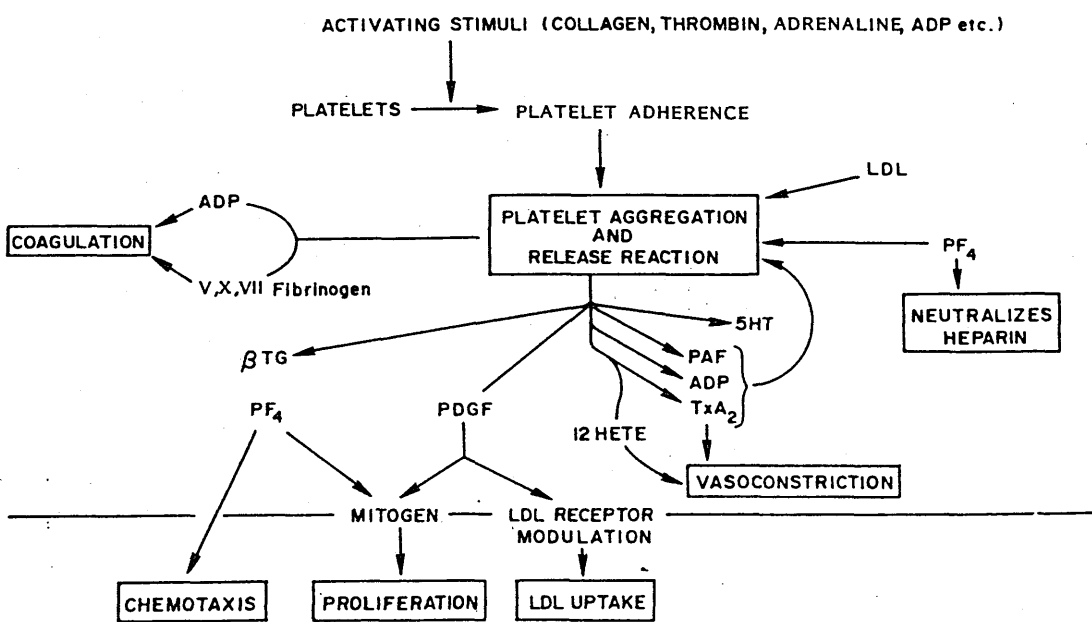
Platelet Adhesion:

Under physiological conditions platelets do not adhere to normal intact endothelium. However, when a blood vessel is injured, the exposed subendothelium rapidly becomes covered by a layer of adhering platelets. The mechanisms underlying platelet deposition on the acutely damaged vessel wall remain poorly understood and appear to be extremely complex. A simplified scheme of the vascular and intravascular events following platelet adherence is shown in Figure 1.7. Adhesiveness is markedly increased by thrombin. The formation of thrombin is initiated at sites of vascular injury by assembly of the prothrombinase complex on the surface of platelets and involves the binding of Factors V and Xa to specific receptors on the platelet membrane (156). Substances present or synthesized in the vessel wall also induce platelet adhesion, particularly ADP, von Willebrand Factor, collagen and

fibronectin (157-161).

FIGURE 1.7

VASCULAR AND INTRAVASCULAR CONSEQUENCES OF PLATELET ACTIVATION



The role of prostaglandins is far from clear. Most investigators have examined the effects in vitro of inhibition of prostaglandin synthesis by aspirin or indomethacin and it is difficult to distinguish the contribution of the different prostanoids and other factors in these experiments (147)(148)(149)(150)(162). The effects of a thromboxane receptor antagonist in an animal model suggest that platelet adhesion is dependent, at least in part, on thromboxane A<sub>2</sub> (163). While some studies have shown inhibition of platelet adhesion by prostacyclin, the concentrations employed appeared to be much greater than those inhibiting platelet aggregation (147) and subsequent work has not confirmed these findings (150).

#### Platelet Aggregation:

Following adhesion to "damaged" endothelium, platelets may then aggregate and release the contents of their granules. Platelet aggregation appears to occur by at least two, possibly three, distinct pathways (164):

- (i) an ADP-induced pathway thought to involve the

interaction of fibrinogen with its platelet receptor (165);

(ii) a prostaglandin dependent pathway mediated by thromboxane  $A_2$  or cyclic endoperoxides binding to the thromboxane  $A_2$ /endoperoxide receptor (99)(101)(102)(103); and

(iii) a third pathway induced by calcium ionophore and thrombin which appears not to be blocked by inhibitors of arachidonic acid metabolism or ADP and which may be mediated by platelet activating factor (166).

ADP and also adrenaline cause a biphasic aggregation response. The second phase or wave of aggregation is associated with liberation of arachidonic acid, which is then metabolized to prostaglandins and thromboxane, and with release of platelet granule contents (104). The prostaglandin pathway may also be initiated directly by metabolism of exogenous arachidonic acid or by a number of other agonists including collagen and thrombin. In low concentrations collagen and thrombin are dependent upon the prostaglandin pathway for their effect. In higher concentrations, however, they appear to be able to stimulate platelet aggregation via several other pathways (164). Responses to all these platelet agonists are

ultimately mediated by changes in intracellular calcium and cyclic AMP. Thus, an increase in the intraplatelet concentration of calcium causes platelet shape change followed by aggregation, whereas an elevation in the level of platelet cyclic AMP, which blocks the mobilization of calcium and lowers the intracellular concentration of calcium, inhibits platelet aggregation (145)(146). This explains the potent platelet inhibitory effects of prostacyclin which causes very marked increases in platelet cyclic AMP levels and blocks the response to all platelet agonists (141).

#### Platelet Secretion:

Platelets contain three classes of secretory granules:  $\alpha$ -granules, dense granules and lysosomes. The  $\alpha$ -granules contain the platelet-specific proteins beta-thromboglobulin, platelet factor 4 and platelet-derived growth factor, as well as thrombospondin which is also synthesized by endothelial cells (167). In addition, homologues of various plasma proteins are present in the  $\alpha$ -granules, including von Willebrand factor, fibrinogen, factor V, fibronectin, plasminogen, high-molecular weight kininogen, albumin and some components of the alternative complement pathway (167).



The dense granules are storage organelles containing calcium, pyrophosphate, 5-hydroxytryptamine and adenine nucleotides. The lysosomes store several acid hydrolases which may be released by high concentrations of thrombin or collagen (168).

The contents of the  $\alpha$ -granules and the dense granules are released in response to platelet activation. Potent agonists such as thrombin and high doses of collagen are able to stimulate platelet secretion by a prostaglandin-independent mechanism (104). However, when weaker agonists, such as adrenaline, ADP and low-dose collagen, are employed, platelet secretion is mediated by the proaggregatory prostaglandins (104). Nevertheless, thromboxane and prostaglandin endoperoxides can also induce platelet aggregation in the absence of secretion (169).

#### Prostacyclin-Thromboxane "Balance" in Haemostasis and Thrombosis:

The discovery of two products of arachidonic acid metabolism, thromboxane  $A_2$  from platelets and

prostacyclin from vascular endothelium, which had potent but opposite effects on platelet function and vascular smooth muscle, led to the hypothesis that the balance between them may be of importance in haemostasis and thrombosis (15)(170). Early work suggested that significant reductions in the "thromboxane  $A_2$ :prostacyclin ratio" were apparent in various animal models of thrombosis (171)(172) and also in human thrombotic disease (173)(174). Furthermore, congenital deficiencies of cyclooxygenase and thromboxane synthase were identified which were associated with haemostatic defects (96)(175)(176). Since that time, however, two principal avenues of research have yielded information which has led to a reevaluation of the concept of a true "balance" between thromboxane  $A_2$  and prostacyclin in vivo.

Firstly, the development of more accurate methods for measuring the biosynthesis of these evanescent compounds has shown that their rate of secretion into the circulation in normal man is extremely low (92)(93)(130). These observations demonstrated that the capacity of tissues to generate icosanoids in response to chemical and physical stimuli greatly exceeds their actual rates of biosynthesis in vivo and suggested that the levels of thromboxane and prostacyclin reported in many of these

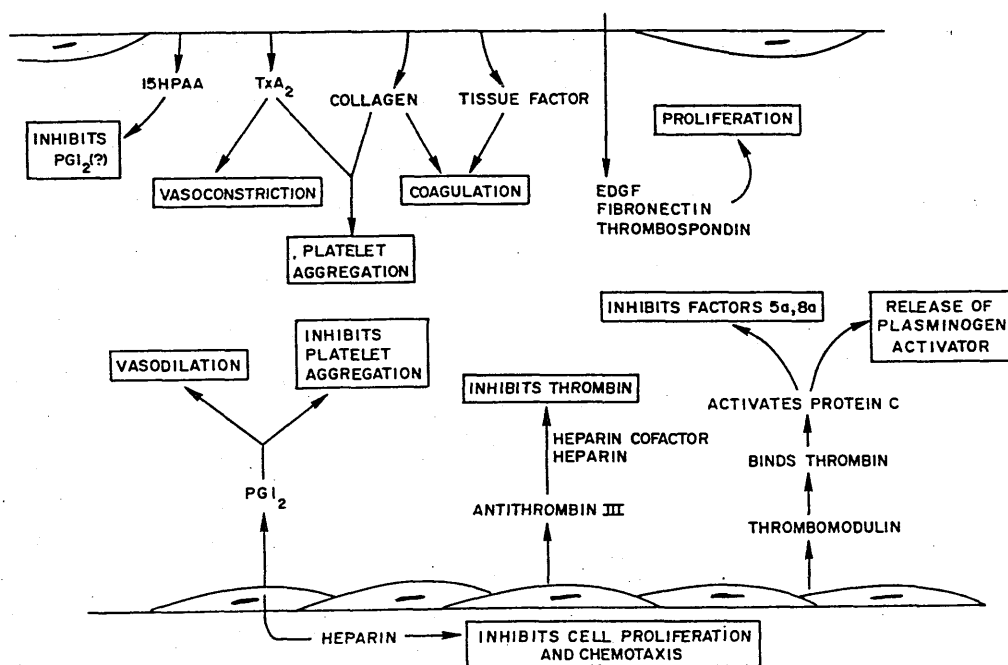
early reports are very likely to have been erroneously high (130). Secondly, not only have a number of new biologically active icosanoids been discovered (18), but there has also been increasing recognition of the biochemical and functional interactions between the different cell types participating in the development of vascular occlusion (177). Thus, while prostacyclin and thromboxane have been confirmed as endogenous compounds with potent biological effects in man, they represent only two of many factors which are important in modifying the development of thrombosis and atherosclerosis (Figure 1.8).

#### Importance of Prostacyclin:

There has been considerable interest in the importance of prostacyclin as a naturally occurring inhibitor of platelet activation both under physiological conditions and in response to diverse physical and chemical stimuli. Its precise role in maintaining vascular endothelium as a non-thrombogenic surface in health and disease states in man is at present unclear. Studies in vitro have shown that atherosclerotic blood vessels have a reduced capacity to synthesize prostacyclin (178)(179). However, subsequent work, has demonstrated that prostacyclin synthesis in vivo

FIGURE 1.8

VASCULAR FACTORS WHICH MODIFY THROMBOSIS AND ATHEROGENESIS



is actually enhanced in patients with severe atherosclerosis who have evidence of platelet activation (180). The authors of this study suggested that such an observation reflected an increased frequency and/or intensity of interactions between activated platelets and the vessel wall. Therefore, although prostacyclin biosynthesis under basal conditions in healthy individuals is apparently insufficient to achieve systemically active concentrations, the enhanced production in severe atherosclerosis may represent an important compensatory response by stimulated endothelium.

Additional evidence in support of the functional importance of prostacyclin in vivo derives from the effects of inhibition of prostacyclin synthesis by aspirin on platelet adherence to vascular endothelium. In a rabbit model of venous thrombosis high doses of aspirin (200mg/kg) increased thrombus formation (182). Similarly, platelet deposition on canine carotid artery vein grafts, pre-treated with aspirin to reduce prostacyclin formation, is enhanced compared with non-aspirin treated grafts (183). Thus, prostacyclin may function mainly as a local modulator of platelet-vessel wall interactions and contribute, along with other endothelial cell-derived products, leucocytes and their products, the physical properties of endothelial cells, haemodynamic elements and

coagulation factors, to the prevention of platelet-dependent thrombus formation at sites of vascular injury.

## THE ROLE OF THROMBOXANE IN HUMAN DISEASE

The potent biological properties of thromboxane have focussed attention on its potential role in the mediation of vaso-occlusive disease. It has been suggested that it may be important in conditions where vascular spasm is known or thought to occur, such as Raynaud's phenomenon (184)(185) and unstable angina (186)(187)(188). In addition, the effect of thromboxane on platelets has led many investigators to implicate thromboxane-dependent platelet activation in the pathogenesis of diverse disease processes in which platelets are thought to play an important role, including atherosclerosis (189-194), the thrombotic complications of myeloproliferative disorders (195)(196), lupus nephritis (197) and the microvascular problems of diabetes mellitus (171)(198-201). A summary of syndromes putatively associated with thromboxane is shown in Table 1.1.

**TABLE 1.1 SYNDROMES PUTATIVELY ASSOCIATED WITH ENHANCED THROMBOXANE SYNTHESIS:**

**Results from Animal Models and Preliminary Studies in Man**

**Ischaemic Heart Disease:**

Coronary thrombosis (203-206)  
Unstable and Prinzmetal's angina (186-8) (207-209)  
Chronic stable angina (209-210)  
Arrhythmias and sudden death (211-216)  
Essential hypertension (217-220)  
Pregnancy-induced hypertension (221-225)

**Cerebrovascular Disease:**

Stroke (226-227)  
Transient ischaemic episodes (228-232)

**Peripheral Vascular Disease (233-234)**

**Renal Disease:**

Immune complex glomerulonephritis (197) (235-237)  
Chronic glomerulonephritis (238-239)  
Hepatorenal syndrome (240)  
Cyclosporin- and adriamycin-induced nephropathy (241-242)  
Renal vein thrombosis (243)  
Allograft rejection (244-245)  
Hypertension following sub-total renal ablation (246-247)  
Hydronephrosis in response to ureteric obstruction (248-249)

**Diabetes Mellitus (171) (198-200)**

**"Collagen-Vascular" Disease:**

Raynaud's phenomenon (184-185)  
Systemic lupus erythematosus (197) (235)

**Myeloproliferative Disorders (195-196)**

**Respiratory Disease:**

ARDS and endotoxic shock (250-256)  
Asthma (257-259)



## Assessing Thromboxane Synthesis and Platelet Activation:

### Methodological Problems

Evaluation of the role played by thromboxane and other arachidonic acid metabolites in the pathogenesis of vaso-occlusive and vasospastic diseases has been confounded by difficulties in measuring the synthesis of these evanescent compounds in vivo (130). The short half-lives, for example, of thromboxane and prostacyclin, necessitated the development of methods to measure their major metabolites rather than the parent compounds (259). Using this approach plasma levels of thromboxane  $B_2$  and the prostacyclin metabolite 6-keto-PGF $_{1\alpha}$  were initially reported to be in the order of 100-200 pg/ml (173)(233)(260)(261)(262). Subsequent work established that the true plasma concentration of 6-keto-PGF $_{1\alpha}$  was less than 2-3 pg/ml (181) and that of thromboxane  $B_2$  is likely also to be greatly over-estimated (92).

The reasons for such a large discrepancy are thought to derive partly from the phasic nature of arachidonate metabolite production (occurring in response to a stimulus rather than continuous biosynthesis) and also from the influence of ex vivo artifact due to invasive sampling techniques. Such a phenomenon is well known to occur with

the introduction of catheters for cardiac catheterization (263)(264). In addition, if care is not taken to minimize ex vivo platelet activation, the conventional methods used to detect platelet activation in vivo yield markedly elevated plasma concentrations of platelet proteins in the absence of true platelet granule release in vivo (265).

An alternative method of assessing thromboxane formation in vivo, which removes the confounding influence of ex vivo platelet activation, is to measure the urinary metabolites of thromboxane. Sensitive and specific assays for the urinary metabolites of prostacyclin have already been developed which have been shown to accurately reflect prostacyclin synthesis in vivo (131)(132). Preliminary evidence suggests that such an approach may be useful for the evaluation of thromboxane formation in vivo (92)(93)(266). The development of accurate methods to measure endogenous biosynthesis of thromboxane is clearly essential for the critical appraisal of pharmacological approaches to the inhibition of thromboxane production in man and for the identification of appropriate clinical targets towards which to direct such therapeutic strategies.

## APPROACHES TO INHIBITION OF THROMBOXANE FORMATION IN MAN

Thromboxane formation can be inhibited by pharmacological agents which block the enzymes in the arachidonic acid cascade directly involved in thromboxane synthesis. The best known of these agents is the cyclo-oxygenase inhibitor, aspirin, which has been used in many large scale clinical trials for the prevention of vascular occlusive syndromes (229)(230-232)(267-273). The major theoretical disadvantage of aspirin in this setting is the reduction in prostacyclin formation which results from concomitant inhibition of the cyclo-oxygenase enzyme in vascular endothelium (Figure 1.9). An alternative approach is to selectively inhibit thromboxane synthase by a specific thromboxane synthase inhibitor (274)(275) (Figure 1.10). There are now several different groups of compounds available which have been shown in vitro to selectively inhibit thromboxane synthesis without depressing prostacyclin production (276)(277). A third and novel approach to inhibition of thromboxane formation was suggested by observations in Greenland Eskimos who were found to have a lower incidence of death from ischaemic

FIGURE 1.9 INHIBITION OF CYCLOOXYGENASE : EFFECTS ON PLATELET VESSEL WALL INTERACTIONS.

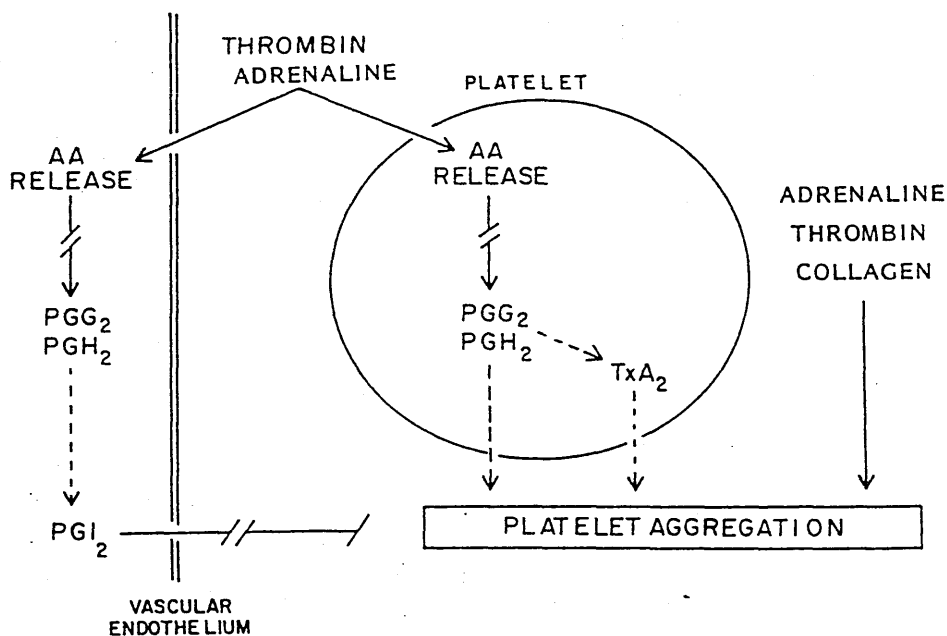
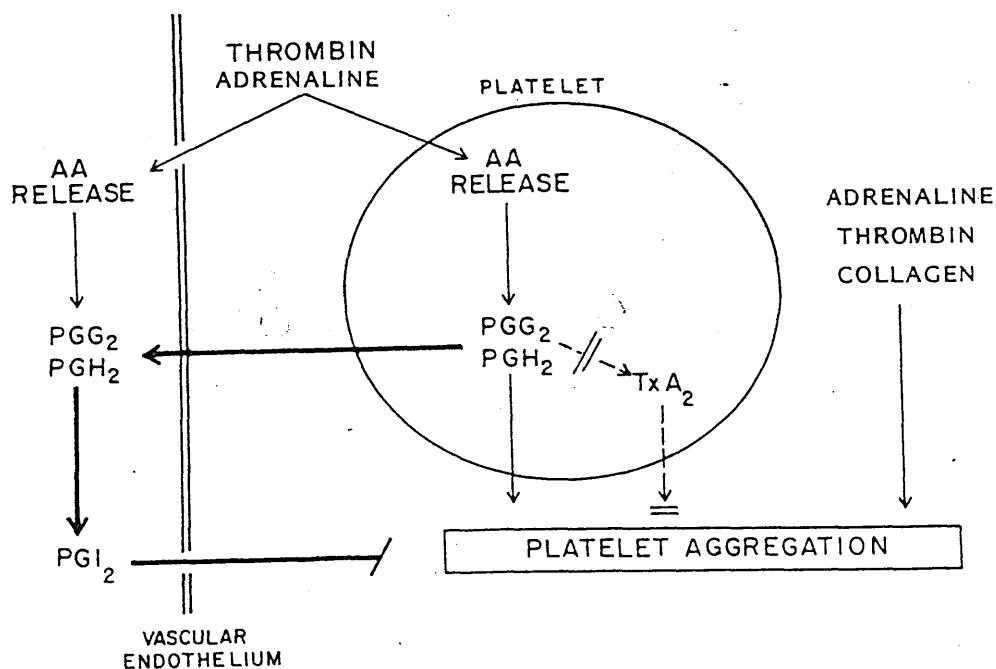


FIGURE 1.10 INHIBITION OF THROMBOXANE SYNTHASE : EFFECTS ON PLATELET-VESSEL WALL INTERACTIONS.



heart disease than Danes (278)(279). This has been attributed to a diet rich in marine oils containing long chain polyunsaturated fatty acids. The principal active constituent appeared to be eicosapentaenoic acid (EPA) which, when given as a dietary supplement, partially replaces arachidonic acid in plasma and platelet lipids (280)(281).

### 1. Inhibition of Enzymes in the Arachidonic Acid Cascade

#### CYCLOOXYGENASE

##### Irreversible Inhibition:

In 1971 three papers were published simultaneously which reported that non-steroidal anti-inflammatory drugs inhibited prostaglandin synthesis in guinea pig lung, human platelets and spleen (5)(6)(282). Roth et al showed that aspirin exerted its platelet-inhibitory effect by binding irreversibly to the cyclooxygenase enzyme (283)(284) and acetylating a serine residue at its active site (285). Thus, as the anuclear platelet is incapable of de novo protein synthesis, aspirin causes irreversible inhibition of prostaglandin endoperoxide and thromboxane

$A_2$  formation for the duration of its lifespan, normally 8-9 days (286).

Aspirin is not only highly active against platelet cyclooxygenase, but also acetylates the enzyme in other tissues, including gastric mucosa, the kidney and vascular endothelium. Thus, coincident with the inhibition of thromboxane  $A_2$  biosynthesis, aspirin has been shown to significantly reduce prostacyclin generation in cultured endothelial cells (287), in isolated vascular segments (288)(289)(290) and in vivo (291). Inhibition of gastric cyclooxygenase causes a marked reduction in  $PGE_2$  and prostacyclin (292) and may play a role in the gastric mucosal damage induced by aspirin. Aspirin also inhibits renal prostaglandin synthesis and may cause significant impairment of renal function under certain conditions (293).

In contrast to the prolonged inhibitory effects on platelets, there is a much more rapid rate of recovery of endothelial cyclooxygenase after exposure to aspirin, perhaps as a result of resynthesis of the enzyme (287)(294). In addition, vascular cyclooxygenase has been shown in some studies to be less sensitive to aspirin in comparison with the cyclooxygenase of platelets (295)(296), although others have failed to confirm this

finding (287). These observations have led to the suggestion that aspirin exhibits dose-related selectivity with respect to its inhibitory effects on prostacyclin and thromboxane formation. Thus, while higher doses depress synthesis of both prostacyclin and thromboxane, it should theoretically be possible to determine a dose of aspirin at which significant inhibition of thromboxane will be achieved in the absence of a fall in prostacyclin production.

#### Reversible Inhibition:

Other non-steroidal anti-inflammatory drugs, for example indomethacin, ibuprofen, naproxen, sulindac and diflunisal (6)(238)(297) inhibit cyclooxygenase in a reversible manner. In addition, sulphinyprazole is a weak inhibitor of cyclooxygenase, the effect persisting only while the drug or its active metabolites remain in the systemic circulation (298)(299). Sulphinyprazole, indomethacin and diflunisal have also been shown to prevent the inhibitory action of aspirin on cyclooxygenase (300). This, and other evidence from the described interaction between aspirin and salicylate, suggests that there are two binding sites on platelet cyclooxygenase: an active or catalytic site and a supplementary site which

regulates, but is not directly involved with, the active site (301).

### THROMBOXANE SYNTHASE

Thromboxane synthase has been partially purified by Ullrich and Haurand using affinity chromatography (302). Their work also suggests that the enzyme, like prostacyclin synthase, is a cytochrome P-450-type haemoprotein. Several classes of compounds have now been developed which are able to selectively inhibit the thromboxane synthase enzyme (274-277). These drugs were designed both as pharmacological tools to investigate the pathophysiological role of thromboxane  $A_2$  and as a potentially useful approach in the treatment of those vaso-occlusive disorders in which thromboxane was thought to be an important factor.

Three classes of thromboxane synthase inhibitors have been described (303)-:

(i) endoperoxide/thromboxane  $A_2$  structural analogues, eg. 9,11-azaprostadienoic acid;



(ii) imidazole and its analogues, eg. dazoxiben (UK 37,248); and

(iii) pyridine derivatives, eg. OKY 1581.

In animal studies and in tissue preparations thromboxane synthase inhibitors have been shown to selectively and reversibly inhibit thromboxane formation (254)(277)(304)(305)(306). They exert no significant inhibitory effects on vascular endothelial cyclooxygenase or on prostacyclin synthase and therefore do not inhibit prostacyclin production. Indeed, a major theoretical advantage of these compounds derives from their potential to enhance prostacyclin biosynthesis as a result of redirection of platelet-derived cyclic endoperoxides to a source of prostacyclin synthase, such as vascular endothelium (62).

Preliminary studies in man show that several of these drugs inhibit the capacity of platelets to generate thromboxane ex vivo in healthy individuals (64)(65)(307-310). There is also evidence that synthesis of prostacyclin may be increased by thromboxane synthase inhibitors in vivo (64)(65). Their effects on platelet function, however, have been variable and may be

relatively minor in normal individuals (307)(309). The ability of these drugs to inhibit thromboxane formation in vivo is investigated for the first time in the following studies. In addition, the biochemical and functional consequences of thromboxane synthase inhibition are evaluated in a clinically relevant model of platelet activation in which endogenous synthesis of thromboxane is markedly enhanced.

## 2. Alternative Fatty Acid Substrates

Although arachidonic acid is the most common fatty acid precursor of prostaglandins in membrane phospholipids, prostaglandins can also be synthesized from alternative fatty acid substrates. Dihomo- $\gamma$ -linolenic acid (eicosatrienoic acid) and eicosapentaenoic acid (EPA) have three and five double bonds respectively instead of the four double bonds present in arachidonic acid. These alternative substrates are metabolized via the same pathways as arachidonic acid. Two double bonds are lost during the cyclooxygenase reaction and therefore eicosapentaenoic acid (EPA) gives rise to prostaglandins of the 3-series, for example  $\text{PGG}_3$ ,  $\text{PGH}_3$ ,

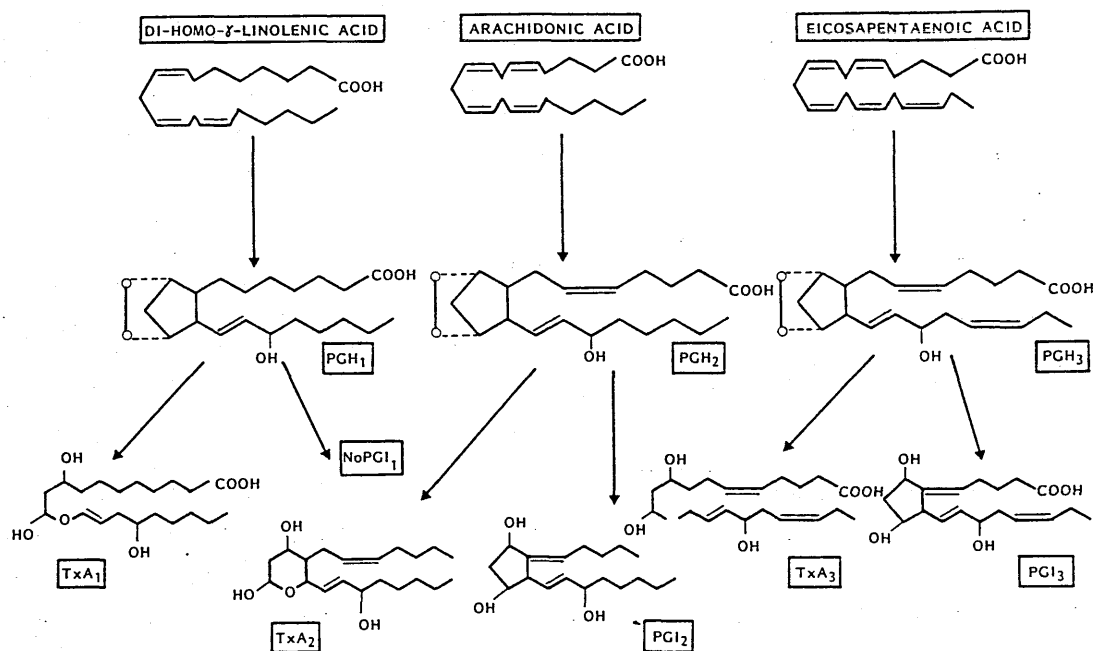
$\text{PGI}_3$  and thromboxane  $\text{A}_3$  (311)(312). Similarly, dihydro- $\gamma$ -linolenic acid is the substrate for prostaglandins of the 1-series, such as  $\text{PGG}_1$ ,  $\text{PGH}_1$  and thromboxane  $\text{A}_1$  (87) (Figure 1.11).

There may be substantial differences, however, between these alternative pathways and the arachidonic acid cascade, both with respect to the affinity of the enzymes for the different substrates and the biological properties of the resultant eicosanoids. Studies of cellular metabolism in vitro have suggested that the products of EPA metabolism were either formed in smaller amounts or were much less biologically potent than the corresponding arachidonic acid derivatives (311)(313)(314). Thus, for example, EPA has been shown in vitro to be a poor substrate for platelet cyclooxygenase (315). Similarly, its analogous product, thromboxane  $\text{A}_3$ , lacks the biological potency of thromboxane  $\text{A}_2$  (311). On the other hand  $\text{PGI}_3$  appears to possess similar biological properties to prostacyclin in that it inhibits platelet aggregation and relaxes vascular smooth muscle in vitro (311).

There has been considerable interest in the possibility of dietary supplementation with fish oils rich in EPA and other omega-3 (N-3) fatty acids as an approach to reducing

FIGURE 1.11

ALTERNATIVE FATTY ACID SUBSTRATES FOR PROSTAGLANDIN SYNTHESIS



the biosynthesis of thromboxane  $A_2$ . A reduction in thromboxane  $A_2$  generation could occur as a result either of a decrease in available arachidonic acid substrate, or of competition by EPA with arachidonate for cyclooxygenase or by increasing the formation of relatively inactive thromboxane  $A_3$  at the expense of thromboxane  $A_2$ . There have now been several studies of the effects of fish oil administration in man (313)(316-323). The principal constituent of the fish oils used, which contain mixtures of fatty acids, is EPA but significant concentrations of docosahexaenoic acid (DCHA) are also present. Preliminary evidence from these studies shows that the capacity of platelets to form thromboxane  $B_2$  ex vivo is diminished during EPA feeding in healthy individuals (316)(317)(319). In addition, prostacyclin biosynthesis appears to be unaltered (312). A recent study by von Schacky and Weber has demonstrated that DCHA not only has intrinsic platelet-inhibitory properties, but that it can be retroconverted into EPA in vivo and may therefore contribute significantly to the putative anti-thrombotic effects of EPA (324). In addition, fish oil has been shown to exert effects distinct from those on platelet eicosanoid formation which may confer additional advantages in the prevention of atherosclerosis (325)(326).

## SUMMARY AND SPECIFIC OBJECTIVES

The pathways of arachidonic acid metabolism in platelets, leucocytes and endothelial cells have been described and the biological effects of the major metabolites in these and other tissues discussed. The principal product of arachidonic acid in human platelets, thromboxane  $A_2$ , is the most potent endogenous platelet agonist known and a powerful vasoconstrictor. It has therefore been implicated in the pathogenesis of a variety of diseases in which platelet activation and vasospasm are believed to be important.

The studies described in this thesis were designed to fulfill the following specific objectives:

1. To investigate the effects on endogenous thromboxane and prostacyclin formation of different approaches to selective inhibition of thromboxane synthesis in healthy individuals.

2. To characterize endogenous biosynthesis of thromboxane in syndromes in which thromboxane-dependent platelet activation is thought to play an important role and in this way identify groups of patients for which this therapeutic strategy is likely to be most appropriate.

3. To examine the biochemical and functional consequences of selective inhibition of thromboxane synthesis in such patients.

## Chapter 2

### METHODS



## I. PROSTAGLANDIN METABOLITES

### General Considerations

#### THROMBOXANE:

Serum Thromboxane B<sub>2</sub>

Urinary 2,3-dinor-thromboxane B<sub>2</sub> (Tx-M):

Sample collection

Internal standard

Methoxime derivatization and extraction

Further purification and derivatization

Gas chromatography/mass spectrometry

Evidence that Tx-M derives from platelets

Urinary Thromboxane B<sub>2</sub>

Calculations

#### PROSTACYCLIN:

Urinary 2,3-dinor-6-keto-PGF<sub>1α</sub> (PGI-M):

Sample collection

Internal standard

Derivatization and purification

Gas chromatography/mass spectrometry

## II. PLATELET FUNCTION

### General Considerations

#### Bleeding Time

#### Platelet Aggregation:

- Preparation of platelet-rich plasma

- Aggregometry

- Platelet aggregation in whole blood

#### Platelet Granule Proteins:

- Beta-thromboglobulin:

- Sample collection

- Radioimmunoassay

- Platelet factor 4

#### Circulating Platelet Aggregates

## III. ASSAYS OF DRUGS AND THEIR METABOLITES

### Aspirin and Salicylate:

- Sample preparation

- Analytical procedure

- Gas chromatography/mass spectrometry

3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid (UK 38,485)

Imidazo(1,5-2)pyridine-5-hexanoic acid (CGS 13080)

#### IV. STATISTICAL METHODS

## I. PROSTAGLANDIN METABOLITES

### General Considerations

Sensitive and specific methods to measure the biologically active eicosanoids are essential, not only for our understanding of their role in health and disease but also to critically evaluate the effects of drug regimens designed to modify their synthesis. The techniques used to quantitate the synthesis of the two most potent eicosanoids, thromboxane  $A_2$  and prostacyclin, are considered here.

The development of methods to measure thromboxane  $A_2$  and prostacyclin has been a problem for many reasons. Firstly, the normal physiological concentration of these compounds appears to be extremely low: certainly less than 5 pg/ml in plasma (92)(93)(130)(132)(181)(327)(328). Highly sensitive assays are therefore required to measure their formation under basal conditions. Secondly, the biological half-lives of thromboxane  $A_2$  and prostacyclin are very short and they are rapidly cleared from the circulation (8)(127). For this reason most of the techniques employed quantitate

their major metabolites rather than the parent compound. Thirdly, several studies have shown that samples obtained using invasive methods, including careful venepunctures and sampling from indwelling cannulae and catheters, are very likely to have falsely elevated values for thromboxane and prostacyclin production (263)(264)(329). This is due to the formation of arachidonate metabolites ex vivo during the sampling procedure and can be reduced by collecting samples immediately into an inhibitor or circumvented by measuring the major metabolites in urine (93)(130)(330). Finally, many other substances are present within the biological fluids being analysed, which may lead to non-specific interference with the assays used to measure the eicosanoids (259).

Three basic approaches to the analysis of human thromboxane and prostacyclin synthesis have been investigated: bioassays (8)(10)(331), radioimmunoassays (328)(330)(331) and stable isotope dilution assays employing gas chromatography/mass spectrometry (GC/MS) (259). Although bioassays have the advantage of detecting compounds with demonstrable biological activity, they are also non-specific and relatively insensitive (331). Radioimmunoassays have been developed for the non-enzymatic hydration products of thromboxane  $A_2$  (thromboxane  $B_2$ ) and prostacyclin (6-keto-PGF $_{1\alpha}$ )

in plasma. Many of the early studies measured levels of these metabolites by radioimmunoassay of several hundred picograms (173)(233)(260)(261)(262), thus greatly over-estimating their true values. This probably reflects a combination of the problems referred to above, including sampling artifacts, as well as cross-reactivity of the antibodies employed and the presence of impurities (130)(332). Although these difficulties have largely been overcome by using highly specific antibodies and rigorous sample purification (328)(333)(334), the problem of sampling artifact remains.

GC/MS was chosen as the best technique for assessing endogenous biosynthesis of thromboxane and prostacyclin in the following studies. It permits the accurate quantitation of the major metabolites of thromboxane and prostacyclin in urine and thus avoids the problem of sampling artifact and ex vivo formation of these metabolites. In addition, GC/MS is highly specific and the most sensitive methods will detect levels of eicosanoid metabolites in the subpicogram range (335). The principal disadvantage of this approach is that it is not possible to determine with certainty the tissue of origin of the measured metabolites. Nevertheless, this can be largely circumvented by measuring the different metabolites in the same urine samples, as discussed below. The use of

timed-aliquot urine collections also facilitates the study of changes in endogenous thromboxane and prostacyclin production over prolonged periods of time.

### THROMBOXANE

Two different approaches to quantitating thromboxane synthesis have been utilized in the following studies:

(i) Measurement of platelet thromboxane formation ex vivo (serum thromboxane  $B_2$ ).

Thromboxane  $B_2$  in serum was measured by radioimmunoassay. This provides an index of thromboxane generation ex vivo and reflects the capacity of platelets to form thromboxane in clotting blood, largely in response to thrombin (336).

(ii) Measurement of thromboxane synthesis in vivo (urinary Tx-M and thromboxane  $B_2$ ).

Endogenous biosynthesis was determined by measuring urinary metabolites of thromboxane. Previous work had

already established that a major metabolite of thromboxane  $B_2$  in urine is 2,3-dinor-thromboxane  $B_2$  (Tx-M) (91) and a method for measuring this metabolite has been described in preliminary studies (266)(291). I utilized a modification of this method for the studies carried out in my thesis. In contrast to the original method, which used electron-impact GC/MS and required extensive purification of large sample volumes, the modification of Lawson et al employs capillary column gas chromatography with negative-ion chemical ionization mass spectrometry and can be applied to samples of 5ml or less (337). The principal innovation is the use of a bonded phase phenyl boronic acid column to selectively extract thromboxanes. This eliminates three high-pressure liquid chromatography steps which were required in the original assay and greatly improves the recovery of the metabolites. It is consequently both specific and sensitive, allowing quantitation of Tx-M in the low picogram range. It can therefore be used to accurately assess the effects of inhibitors of thromboxane synthesis on endogenous thromboxane formation. In addition, it permits the concomitant measurement of urinary thromboxane  $B_2$ . In contrast to the dinor metabolite (Tx-M), urinary thromboxane  $B_2$  is thought to be mainly of renal origin under physiological conditions (338) and measurement of both metabolites may be useful in



distinguishing between platelet and renal thromboxane biosynthesis.

### Serum Thromboxane B<sub>2</sub>

Venous blood (1-2ml) was collected via a 19-gauge butterfly needle into a warmed glass tube, allowed to clot and incubated at 37°C in a water bath for 45-60 minutes. The sample was centrifuged at 2,000g for 15 minutes and the serum was separated and stored at -20°C for later analysis. Radioimmunoassay was performed as described by Patrono et al (336) using an antibody generously provided by Dr RJ Workman (Vanderbilt University). Serial dilutions (1:25 - 1:625) of the unextracted serum were prepared in duplicate in phosphate buffer and assayed at a final dilution of 1:500 - 1:12,500. The assay used <sup>125</sup>I-thromboxane B<sub>2</sub> and a rabbit anti-thromboxane B<sub>2</sub> antibody which were added to each sample and incubated at 0°C for 60-90 minutes. After addition of polyethylene glycol (21%) and centrifugation at 2,000g for 15 minutes at 0°C, the fluid phase was removed and the radioactivity of the sedimented fraction counted.

The detection limit of the assay was 1 ng/ml. Normal values ranged between 119 and 779 ng/ml (mean 366 +/- 33 ng/ml) and were unaffected by age or sex (see Appendix I). As reported by others (339)(340), there was a positive correlation between platelet count and serum thromboxane  $B_2$  generation ( $r=0.588$ ;  $p<0.001$ ) in normal subjects. The contribution of platelet number to the overall variation in serum thromboxane  $B_2$  observed in healthy individuals has been shown to be minor and insufficient to warrant correction of serum levels for platelet count (339).

Urinary 2,3-dinor-thromboxane  $B_2$  (Tx-M) and  
Thromboxane  $B_2$

Sample collection:

Urine was collected in plastic containers with no added preservative. Baseline samples were usually collected over a 24-hour period, but shorter (6 or 12-hour) collections were used to assess the effects of inhibitory therapy in order to detect transient changes in metabolite excretion. In every case concomitant measurement of urinary

creatinine was carried out to correct for variations in urine volume and the possibility of incomplete collections. Whenever possible the containers were kept at 4°C during the collection.

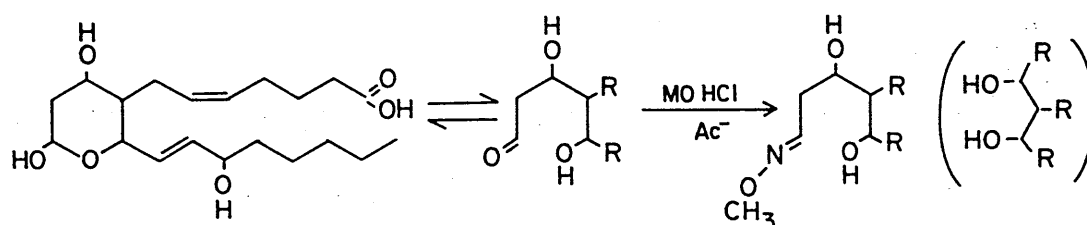
#### Internal Standard:

Tx-M was measured using a stable isotope dilution assay as described by Lawson et al (337). Deuterated 2,3-dinor-thromboxane  $B_2$  (19,19,20,20- $^2H_4$ -dinor thromboxane  $B_2$ ) provided by Drs F. Fitzpatrick and J. Pike (Upjohn Company, Kalamazoo, Michigan, USA) was used as the internal standard. A known amount of internal standard (2.5 ng/ml of urine) was added to each aliquot of urine as soon as possible after collection and these "spiked" urine samples were stored at -20°C until later analysis. Samples could be stored in this way for several months without any significant loss of activity and the addition of the internal standard soon after collection ensured that any change occurring with storage affected the standard and endogenous metabolite equally.

### Methoxime Derivatization and Extraction:

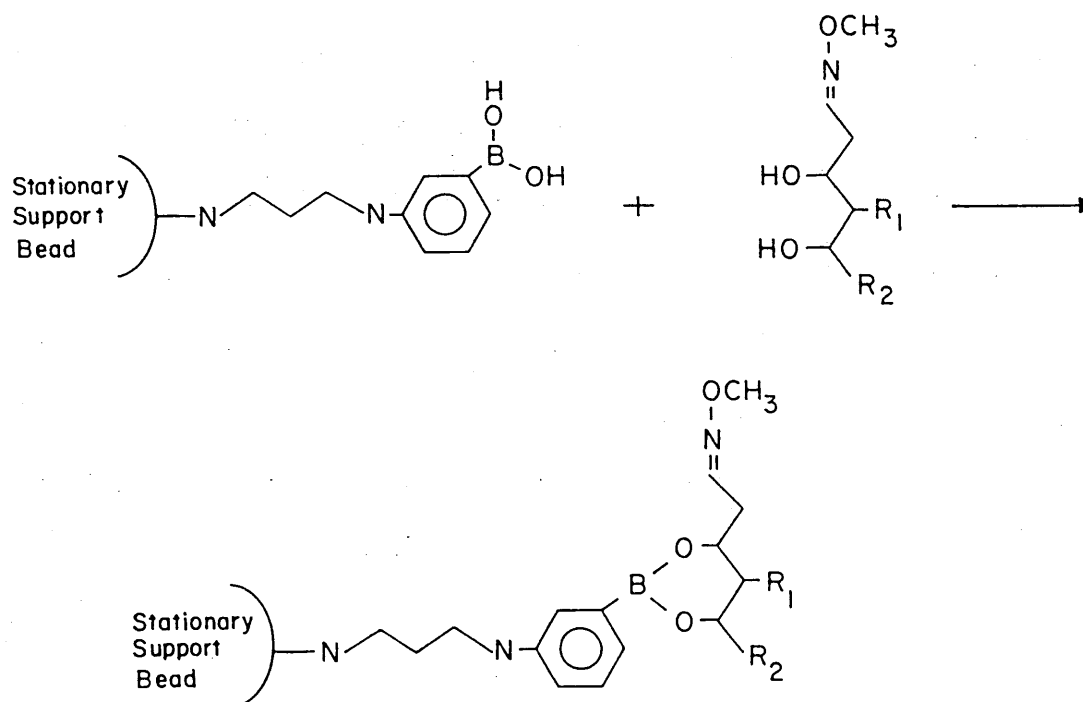
A 5ml aliquot of "spiked" urine was thawed and mixed with 0.5g methoxyamine hydrochloride and 1.5ml acetate buffer and allowed to stand at room temperature for at least 5 minutes to form the methoxime derivative (Figure 2-1). The sample was then applied to a phenylboronic acid column which had been prepared with 2ml methanol and converted to the trigonal neutral form with 0.1N hydrochloric acid. Formation of the methoxime derivative with its open ring structure is essential for efficient bonding of Tx-M to the phenylboronic acid column (Figure 2-2). The column was then rinsed with acid salt followed by methanol and the metabolite was eluted using 0.1N sodium hydroxide in methanol. The eluate was acidified with 10% formic acid and purified by application to a reverse-phase Sep-Pak cartridge which had been primed with methanol then distilled water. The column was washed with distilled water followed by methanol:water (1:3) and the metabolite was then eluted with ethyl acetate and evaporated to dryness under nitrogen.

FIGURE 2.1 DERIVATIZATION OF Tx-M: METHOXIME FORMATION IN URINE.



### METHOXIME FORMATION IN URINE

FIGURE 2.2 DERIVATIZATION OF Tx-M : BONDING TO THE PHENYLBORONIC ACID COLUMN.



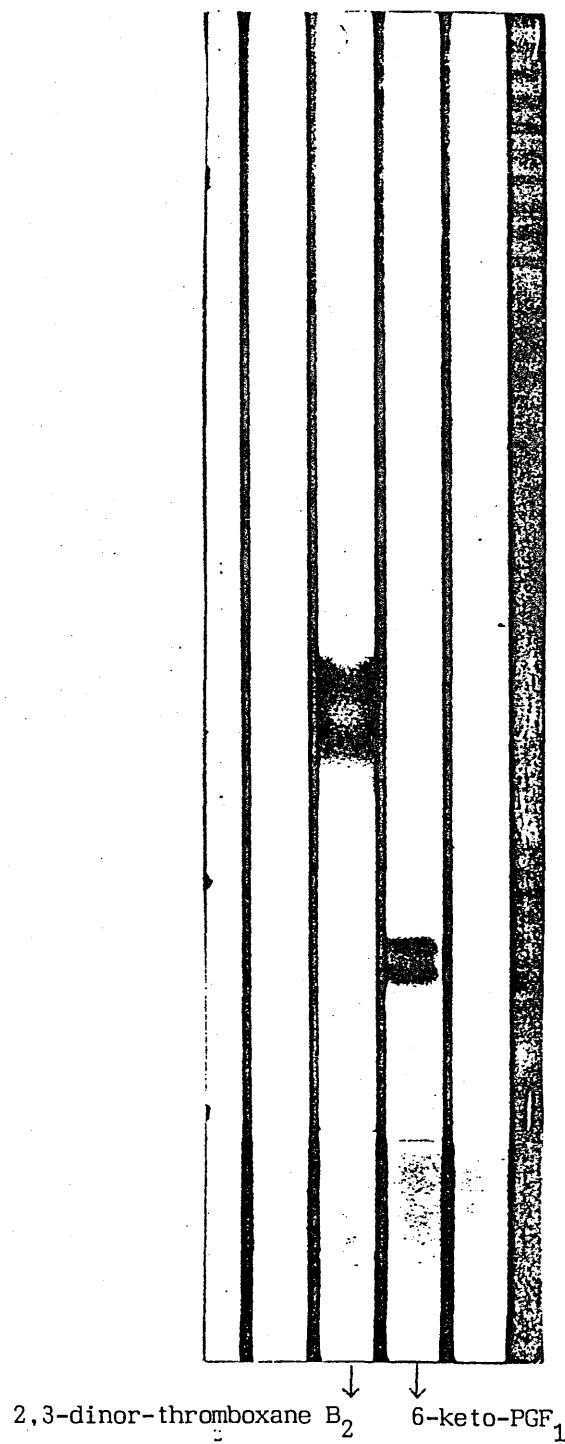
### Further Purification and Derivatization:

The dried extract from the reverse-phase cartridge was dissolved in methanol, applied to a silicic acid thin layer chromatography plate and run in a solvent system of ethyl acetate, acetic acid and hexane (54:12:25, vol:vol:vol). Unlabelled 2,3-dinor-thromboxane B<sub>2</sub>-methoxime run concurrently on a separate plate was used as the standard (R<sub>f</sub> approximately 0.21). For each sample, the area of the plate corresponding to the R<sub>f</sub> value of the standard was scraped (Figure 2-3), added to 1.5ml ethyl acetate and, after centrifuging for 1 minute at 900rpm, the organic layer was removed and evaporated to dryness under nitrogen.

The sample was further derivatized as the pentafluorobenzyl ester by adding di-isopropylethylamine (10ul) and 12.5% pentafluorobenzyl bromide in acetonitrile (20ul) and heating for 30 minutes at 40°C. Derivatization is necessary in order to increase the sensitivity and specificity of the assay by improving the gas chromatography characteristics of the metabolite (259). The pentafluorobenzyl ester derivative was applied to a thin layer chromatography plate and run in a solvent system of isooctane and ethyl acetate (65:85, vol:vol)

FIGURE 2.3

Thin layer chromatogram of authentic standards of  
2,3-dinor-thromboxane  $B_2$  and 6-keto-PG  $F_{1\alpha}$



For each sample a 1-1.5 cm area of the plate corresponding to a concurrently run standard was scraped and extracted into ethyl acetate.

using the same standard as above which had been subjected to derivatization in the same way as the samples. The plate was scraped as before ( $R_f$  value approximately 0.11), added to ethyl acetate and the organic layer aspirated and evaporated to dryness. Final derivatization was achieved by adding bis (trimethylsilyl) trifluoroacetamide (10ul) and pyridine (10ul) to form the trimethylsilyl ether.

#### Gas Chromatography-Mass Spectrometry:

The samples were analysed by gas chromatography-mass spectrometry using a Nermag 10-10C mass spectrometer linked to a Varian Vista 6000 gas chromatograph. The mass spectrometer was operated in the negative ion-chemical ionization mode. The mass spectra obtained in this mode are much simpler than those obtained by electron impact mass spectrometry. In addition, it is also highly efficient and, as the principal fragmentation ions are in the high mass range, there is good separation of Tx-M from any contaminating substances which may remain despite rigorous purification. Negative ion-chemical ionization is therefore both very specific and very sensitive (259). Specificity was augmented by using capillary column gas chromatography (a 5 M DB-1 capillary column) to improve resolution. Selected ion monitoring was carried out at  $m/z$  (mass/charge ratio) 586 for endogenous Tx-M and  $m/z$  590



(ie 4 mass units higher) for the tetradeuterated internal standard. The negative ion-chemical ionization mass spectrum of the methoxime, trimethylsilyl ether, pentafluorobenzyl ester derivative of Tx-M is shown in Figure 2-4. Selected ion monitoring for the same compound is shown in Figure 2-5. The top trace represents endogenous Tx-M and the lower trace the internal standard.

Normal values were established first in young healthy male subjects. These results and the effects of physiological variables on Tx-M excretion are given in Appendix I. Mean daily Tx-M excretion was  $138 \pm 20$  pg/mg creatinine with a range of 73 to 237 pg/mg creatinine. The equivalent values expressed in ng/hour were  $10.3 \pm 2.0$  (range 4.8 to 24 ng/hour). Subsequent experiments showed that there was no difference between male and female subjects. Tx-M was significantly higher in healthy individuals over the age of 50 years ( $223 \pm 22$  pg/mg creatinine), despite normal creatinine excretion. The relationship between age and Tx-M concentration was linear between the ages of 20 and 90 years in healthy subjects.

FIGURE 2.4 NEGATIVE ION-CHEMICAL IONIZATION MASS SPECTRUM OF THE METHOXIME, TRIMETHYLSILYL ETHER, PENTAFLUOROBENZYL ESTER DERIVATIVE OF Tx-M.

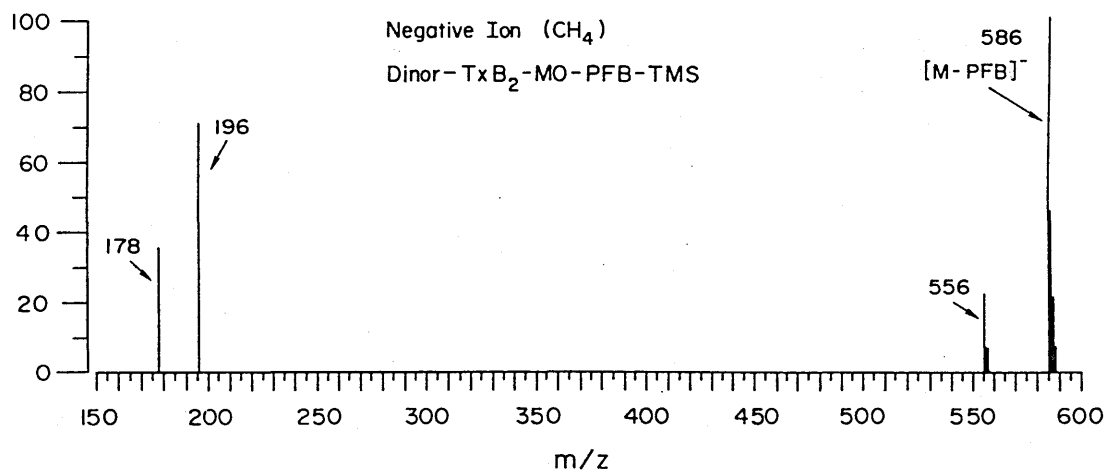
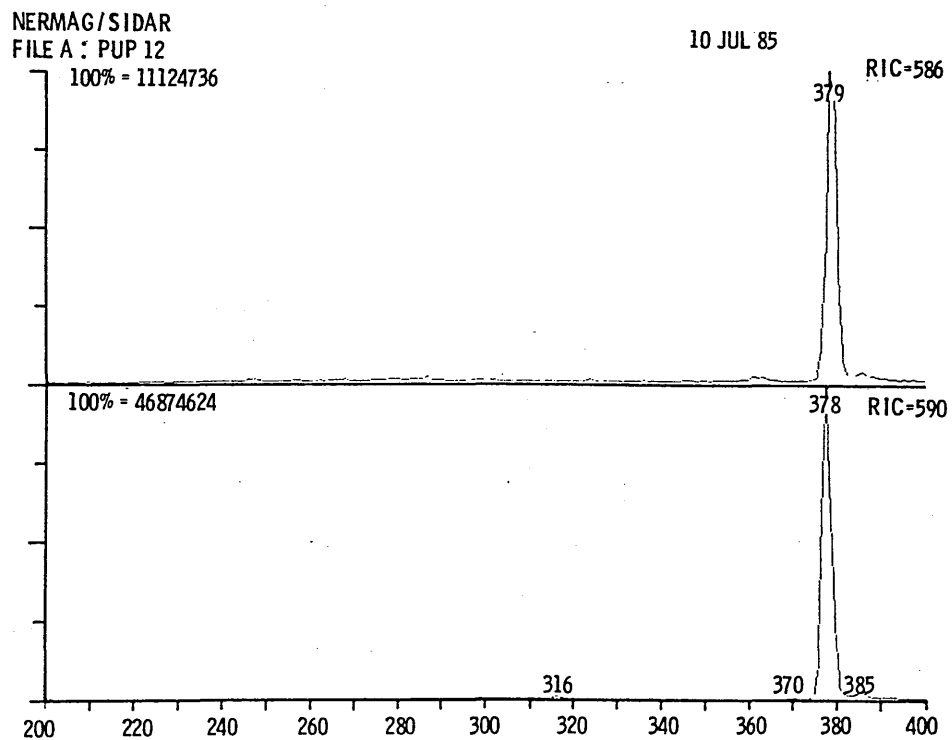


FIGURE 2.5 SELECTED ION MONITORING TRACE FOR THE METHOXIME, TRIMETHYLSILYL ETHER, PENTAFLUOROBENZYL ESTER DERIVATIVE OF Tx-M AND THE TETRADEUTERATED INTERNAL STANDARD.



### Evidence that Tx-M derives predominantly from platelets

The levels of Tx-M measured in urine reflect total body synthesis of thromboxane  $B_2$ . Tx-M is therefore derived from both platelet and extraplatelet sources. In platelets, however, thromboxane  $A_2$  is the principal metabolite of arachidonic acid, whereas in most tissues which have the capacity for thromboxane synthesis, such as the kidney, other metabolites predominate (34)(35). Furthermore, as can be seen from the concentrations of thromboxane  $B_2$  in serum, platelets have a huge capacity for thromboxane generation in response to agonists such as thrombin. Before proceeding to study the biochemical and functional effects of different approaches to inhibition of thromboxane synthesis in detail, I carried out some preliminary experiments to address the hypothesis that urinary Tx-M is predominantly derived from platelets.

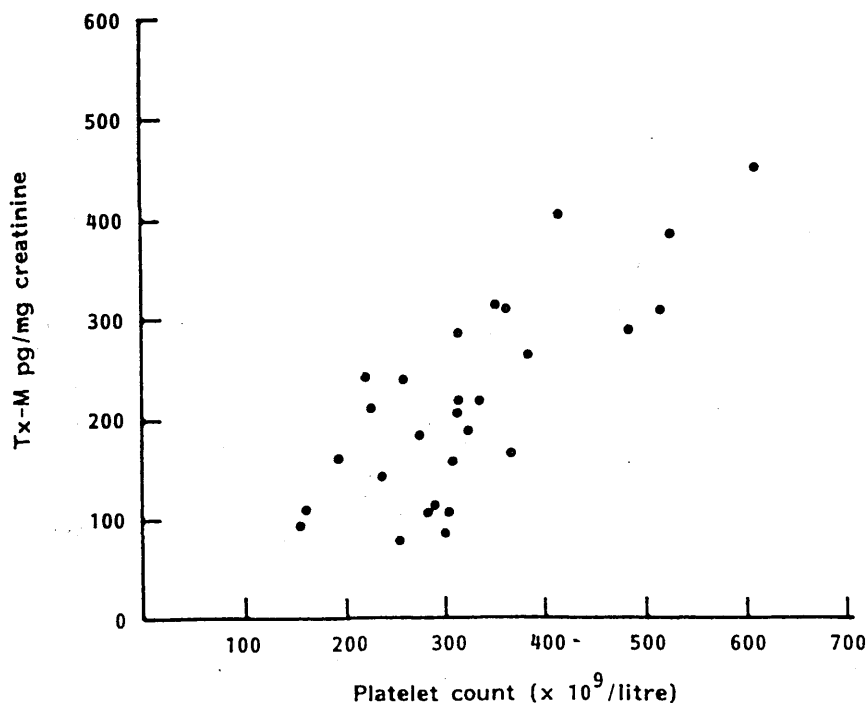
1. Relationship between Tx-M excretion and platelet count:

Method: Tx-M was measured in 24 hour urine collections from 28 healthy subjects aged 21-75 years. Platelet counts were performed in each subject on peripheral venous blood samples analysed on a Coulter counter.

Results: As shown in Figure 2.6, Tx-M was proportional to platelet count over a wide range of counts ( $r=0.91$ ;  $p<0.001$ ).

FIGURE 2.6

RELATIONSHIP BETWEEN Tx-M AND PLATELET COUNT  
IN 28 HEALTHY SUBJECTS



## 2. Effect of increased platelet turnover on Tx-M:

Method: 24-hour urine collections for measurement of Tx-M were made in three patients with clinical syndromes in which platelet turnover is known to be increased. Two patients had immune thrombocytopenia and one (J.B.), in whom measurements were made on 2 separate occasions, had hypersplenism.

Results: The results are shown in Table 2.1. Bone marrow findings on all the patients were consistent with thrombocytopenia due to peripheral platelet destruction with a shortened platelet lifespan. All three patients also had significantly elevated levels of urinary Tx-M.

TABLE 2.1 Tx-M EXCRETION IN PATIENTS WITH INCREASED PLATELET TURNOVER.

Patient	Tx-M (pg/mg creatinine)	Platelet Count ( $\times 10^9/l$ )
JB	1478	63
	1699	69
AR	449	42
MF	1492	109
PE	545	45

3. Tx-M excretion in patients with platelet activation in vivo:

Method: 10 patients with various conditions in which platelet activation in vivo is thought to occur were studied. Tx-M was measured in 24-hour urine collections and in some patients plasma  $\beta$ -thromboglobulin was quantitated by radioimmunoassay as described below. In the patient with acute myocardial infarction serial urine collections were made before and after the event.

Results: The results are shown in Table 2.2. In this small uncontrolled pilot study all patients were shown to have elevated Tx-M excretion (compared to values measured separately in normal subjects), despite normal platelet counts in all but one. The exception was the patient (R.B.) with primary polycythaemia who had mild thrombocytosis. In those patients in whom  $\beta$ -thromboglobulin concentrations were available, these were also increased to a variable extent, consistent with platelet activation in vivo. Serum thromboxane  $B_2$ , which is a capacity-related index and would not therefore be expected to be increased in the presence of platelet activation in vivo, was normal in all the subjects tested.

TABLE 2.2 Tx-M EXCRETION IN PATIENTS WITH PLATELET ACTIVATION IN VIVO.

Patient	Diagnosis	Tx-M (pg/mg creatinine)	$\beta$ -thromboglobulin (ng/ml)	Platelets ( $\times 10^9/l$ )	Serum TxB <sub>2</sub> (ng/ml)
MS	Severe generalized atherosclerosis	1701	39	236	413
AP	Severe peripheral vascular disease	994	48	279	506
SS	Systemic sclerosis with Raynaud's	989	23	NA	323
MR	Systemic sclerosis with Raynaud's	713	53	NA	471
MO	SLE with lupus anticoagulant and recurrent thromboses	408	NA	151	NA
RB	Myeloproliferative disorder (polycythaemia vera)	496	69	738	481
LW	Myeloproliferative disorder (CGL)	823	50	433	439
CD	Atherosclerosis, diabetes, Acute myocardial infarction - before: 1st 24hrs: after 7 days:	1086 2874 1140	NA NA NA	236 271 308	220 309 410

NA : not available

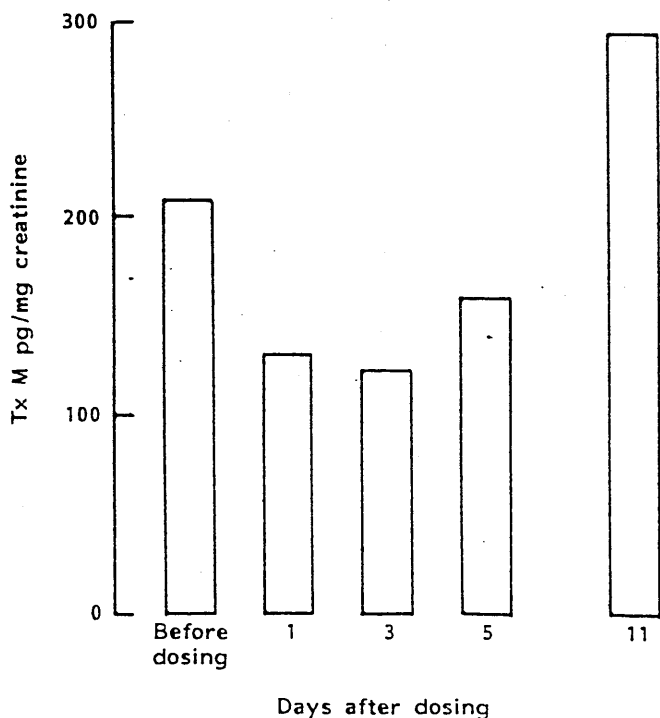
4. Effect of aspirin on Tx-M:- pattern of inhibition and recovery:

Method: A single dose of 650mg of aspirin was given to 3 healthy male volunteers. Urine was collected in 24-hour aliquots before and at intervals for 11 days after aspirin administration.

Results: Tx-M fell following the aspirin and remained inhibited for several days, returning to normal by 11 days after dosing (Figure 2.7).

FIGURE 2.7

INHIBITION AND RECOVERY OF Tx-M AFTER  
SINGLE DOSE ADMINISTRATION OF 650mg ASPIRIN





## 5. Review of other studies:

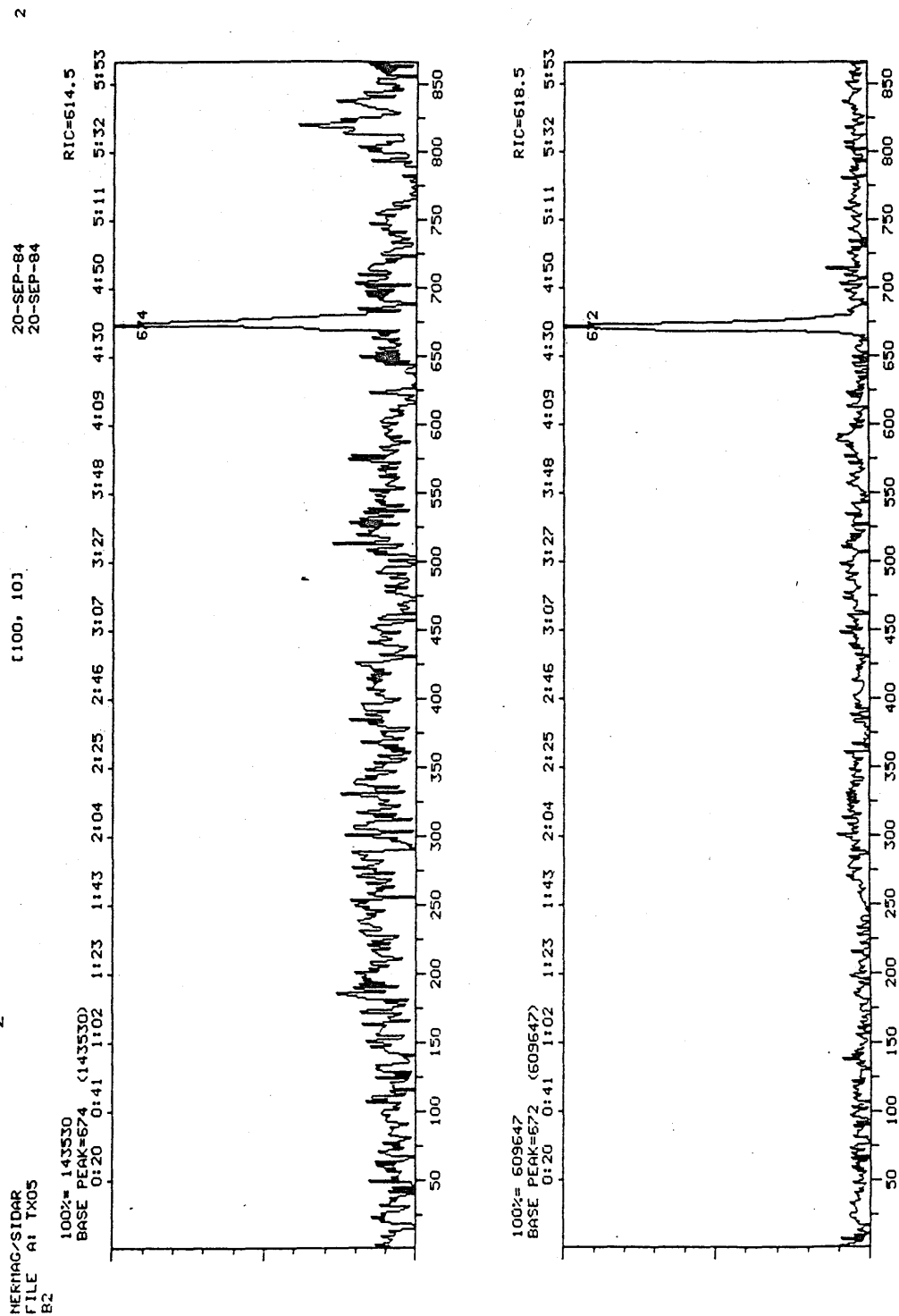
There is also evidence from other studies consistent with a mainly platelet origin for Tx-M. Levels of Tx-M declined by over 90% during chronic administration of high dose aspirin (291). Excretion of Tx-M remained depressed for several days after aspirin was discontinued, consistent with irreversible acetylation of platelet cyclooxygenase. By contrast, the recovery of Tx-M was much more rapid after reversible cyclooxygenase inhibition induced by indomethacin (266). Tx-M is also significantly inhibited after chronic administration of low-dose aspirin (291), whereas urinary thromboxane B<sub>2</sub>, which is believed to be primarily of renal origin under physiological conditions, remains unaltered (338). Finally, Tx-M has been shown to be markedly increased in another syndrome of platelet activation in vivo, thrombotic thrombocytopenic purpura, and to fall towards normal in response to prostacyclin infusion (341).

## Measurement of Urinary Thromboxane B<sub>2</sub>

Urinary excretion of thromboxane B<sub>2</sub> was measured by a similar stable isotope dilution assay. The internal standard was tetradeuterated thromboxane B<sub>2</sub> (generously provided by Drs. Fitzpatrick and Pike, Upjohn Company, Kalamazoo, Michigan). The purification and derivatization steps were identical with those described above for Tx-M with the exception that, since the relative migration rates for thromboxane B<sub>2</sub> and Tx-M (respectively 0.33 and 0.21) are different, the appropriate areas of the thin-layer chromatography plates were scraped at each step of the thin layer chromatography procedure.

Quantitation was established using the Nermag 10-10C instrument operating in the negative ion mode monitoring m/z 614 for endogenous thromboxane B<sub>2</sub> and m/z 618 for the internal standard. A selected ion monitoring trace for endogenous thromboxane B<sub>2</sub> (top trace) and the tetradeuterated standard (bottom trace) is shown in Figure 2.8. Urinary thromboxane B<sub>2</sub> ranged from 16 to 76 pg/mg creatinine in healthy subjects with a mean of 46 +/- 6 pg/mg creatinine. The equivalent values in ng/hour ranged from 0.5 to 7.3 with a mean of 2.8 +/- 0.7 ng/hour

FIGURE 2.8 SELECTED ION MONITORING TRACE OF THE METHOXIME, TRIMETHYLSILYL ETHER, PENTAFLUOROBENZYL ESTER DERIVATIVE OF THROMBOXANE B<sub>2</sub> AND THE TETRADEUTERATED INTERNAL STANDARD.



(see Appendix I).

Calculations:

The urinary excretion of thromboxane  $B_2$  and Tx-M, the dinor metabolite, is expressed in most of the experiments in pg/mg of urine creatinine. The amount of endogenous thromboxane  $B_2$  or Tx-M is calculated from selected ion monitoring traces by comparing the peak height of the endogenous compound with that of the known amount of internal standard. The value obtained in pg/ml is then corrected for the total volume of urine collected and the concentration of creatinine. Correction of Tx-M or urinary thromboxane  $B_2$  for creatinine was particularly important when short timed collections were employed. In experiments in which differences in Tx-M excretion were sought between groups of subjects (eg patients with atherosclerosis versus healthy individuals) the rate of excretion in ng/hour was also determined.

## PROSTACYCLIN

Prostacyclin biosynthesis was determined by measuring urinary 2,3-dinor-6-keto-PGF<sub>1 $\alpha$</sub>  (PGI-M) using a stable isotope dilution assay. The method employed was a modification (335) of the original method described by Falardeau et al (342).

### Urinary PGI-M:

#### Sample collection:

Urine was collected into plain plastic containers. The collections were timed and, as for the thromboxane metabolites, the amount of PGI-M measured was corrected in each case for creatinine excretion.

#### Internal Standard:

Tetradeluterated 2,3-dinor-6-keto-PGF<sub>1 $\alpha$</sub>  (supplied by Drs Fitzpatrick and Pike of the Upjohn Company) was employed as the internal standard for this assay. The

standard was added at a concentration of 1 ng/ml of urine. Aliquots of urine were "spiked" with the internal standard as soon as possible after collection and the samples stored at -20 °C for later analysis.

#### Derivatization and Purification:

After thawing, the "spiked" samples were subjected to extraction and back extraction under acidic and alkaline conditions. The extract was derivatized as the methoxime by addition of 0.5% methoxyamine hydrochloride in pyridine and heating for 60 minutes at 70°C. The samples were then evaporated to dryness under nitrogen and further derivatized as the pentafluorobenzyl ester. This was accomplished by addition of di-isopropylethylamine (10ul) and 12.5% pentafluorobenzyl bromide in acetonitrile (20ul) and heating for 30 minutes at 40°C.

Further purification was carried out by thin-layer chromatography in a solvent system of 2% methanol in ethyl acetate using as standard 6-keto-PGF<sub>1α</sub> derivatized as the methoxime pentafluorobenzyl ester (Rf approximately 0.18). Derivatization was completed by formation of the trimethylsilyl ether by addition of bis (trimethylsilyl) trifluoroacetamide (10ul) and pyridine (10ul).

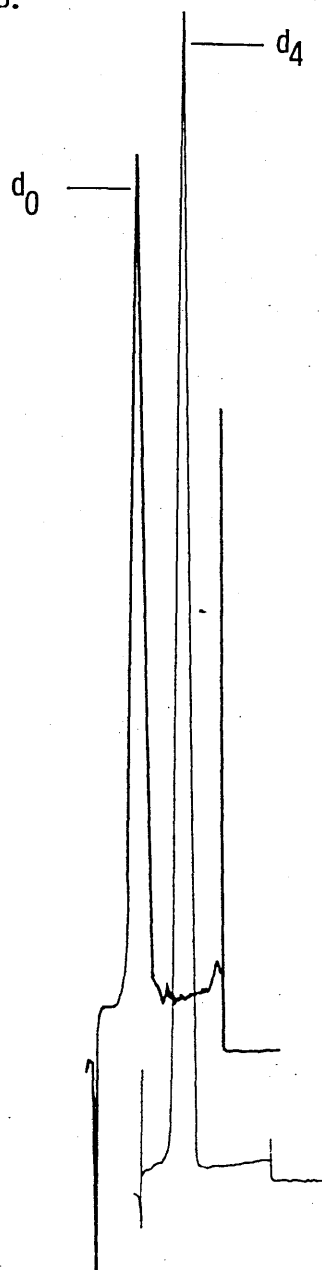
### Gas Chromatography-Mass Spectrometry:

Quantitation was accomplished using a Hewlett Packard 5980 instrument operating in the negative ion-chemical ionization mode. Monitoring was at  $m/z$  586 for endogenous PGI-M ( $d_0$ ) and  $m/z$  590 for the tetradeuterated internal standard ( $d_4$ ). A representative trace is shown in Figure 2.9. Calculation of the amount of PGI-M present was achieved by comparing the measured peak heights of endogenous PGI-M to that of the known amount of internal standard, as for the thromboxane metabolites, and correcting for urine volume and creatinine concentration. The results were mainly expressed in pg/mg creatinine. In some experiments the data is also given in ng/hour.

In control experiments in healthy subjects PGI-M excretion ranged from 55 to 142 pg/mg creatinine with a mean of  $101 \pm 9$  pg/mg creatinine. The equivalent values in ng/hour ranged from 3.6 to 13.8 with a mean of  $8.1 \pm 0.6$  ng/hour (see Appendix I). There was no difference between men and women, but values were higher in older subjects (mean PGI-M in subjects over 50 years:  $197 \pm 21$  pg/mg creatinine), despite normal creatinine excretion

(see Appendix I).

FIGURE 2.9 SELECTED ION MONITORING TRACE OF THE METHOXIME, TRIMETHYLSILYL ETHER, PENTAFLUOROBENZYL ESTER DERIVATIVE OF PGI-M AND THE TETRADEUTERATED INTERNAL STANDARD.





## II. PLATELET FUNCTION

### General Considerations

A variety of approaches to the assessment of platelet function have been developed. Although there are a number of animal models of platelet activation in vivo (203)(343)(344)(345), the skin bleeding time is the only reliable measure of haemostatic function in vivo in man. The bleeding time, however, is relatively non-specific as it is influenced by other factors in addition to platelets (346). Methods to specifically evaluate platelet function have mainly been directed towards their ability to respond to various stimuli in vitro. The most widely used technique, platelet aggregometry by the light transmission method, was described by Born in 1962 (347). Since that time various methods to measure platelet aggregation in whole blood have also been devised in the hope that such an approach would more closely represent the processes occurring in vivo (348)(349). Alternatively, measurement of circulating platelet aggregates and of the platelet-specific proteins,  $\beta$ -thromboglobulin and platelet factor 4 released when platelets are activated, have been employed as markers of platelet activation in vivo in

several disease states (167)(265)(350).

#### BLEEDING TIME

In all of these studies the bleeding time was measured by the template method using the Simplate I device. This device has been shown to give reproducible results and to produce a significantly prolonged bleeding time after aspirin ingestion (351). The test was performed on the volar aspect of the forearm and care was taken to avoid blood vessels. The incision was consistently made perpendicular to the fold of the elbow. A sphygmomanometer cuff was kept inflated at a pressure of 40 mm Hg throughout the procedure. The blood emerging from the incision was removed every 30 seconds with a disc of filter paper until the bleeding stopped. In my hands the mean normal value for the bleeding time using this technique was 5.0 +/- 0.4 minutes in healthy individuals with a range of 2.5-7.0 minutes (see Appendix II).

## PLATELET AGGREGATION

Platelet aggregation in most of the studies was measured in platelet-rich plasma by the light transmission method (347). In some experiments an impedance method was also used to examine platelet aggregation in whole blood (348). The principal advantages of the light transmission method are that it is a well validated method which has been in use for over 25 years and provides reproducible data. The availability of two- and four-channel machines has speeded up the procedure and allows more samples with different agonists to be tested within a short time of collection. It does not, however, take account of the possible contributions of erythrocytes and leucocytes to the process of platelet aggregation and also, as a result of sample preparation, may select a particular subpopulation of platelets for study rather than reflecting the behaviour of the circulating platelet pool. Whole blood platelet aggregometry was developed in an attempt to remedy some of these problems and has the additional advantage of allowing the sample to be analysed within a few minutes of blood withdrawal (348)(352).

#### Preparation of platelet-rich plasma:

Platelet-rich plasma (PRP) was prepared from venous blood collected into 3.8% sodium citrate (1:9 citrate: blood). The citrated blood was transferred to plastic tubes and centrifuged at 150g for 15 minutes at 20°C. The PRP was removed and platelet-poor plasma (PPP) prepared by further centrifugation of the remaining blood at 2,000g for 15 minutes. The platelet count of the PRP was determined using a Coulter counter and adjusted to  $300 \times 10^9/l$  by addition of appropriate quantities of PPP. Aliquots of 0.5 ml PRP were kept at 37°C and tested within 2 hours of collection.

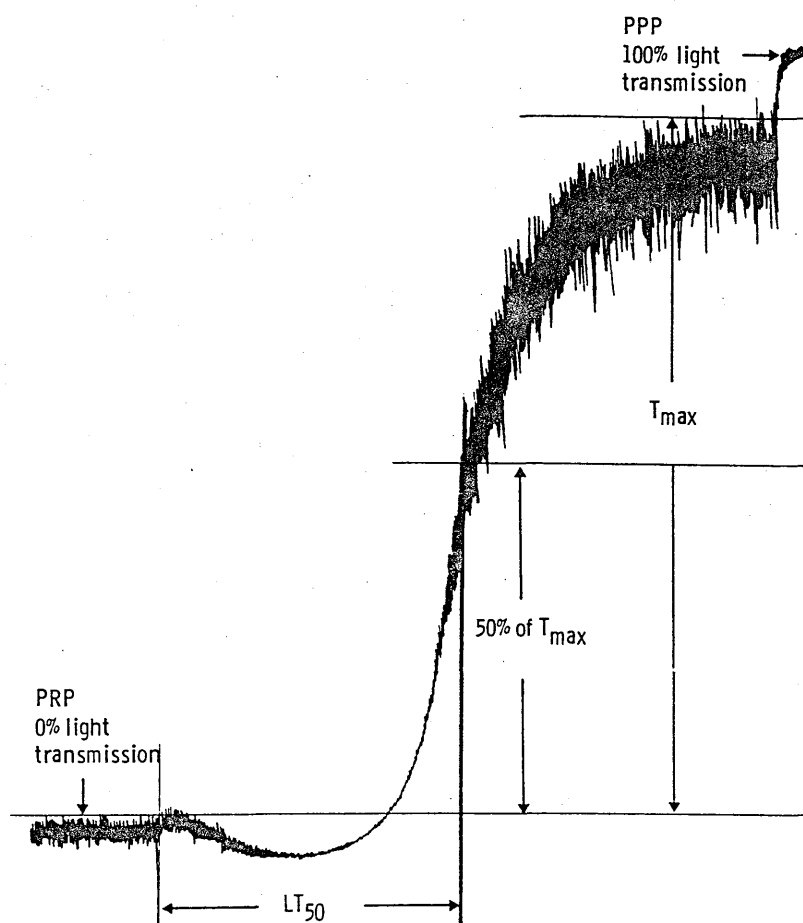
#### Aggregometry:

Platelet aggregation was measured in a Payton dual-channel aggregometer. The agonists used were: collagen in a high dose (19 ug/ml, final concentration) and a low dose (1.9 ug/ml); arachidonic acid in various concentrations from 0.33-1.33 mM; and adrenaline 1-10 uM. All the reagents were freshly prepared on the day of study. In each case the sample of PRP was preincubated for 1 minute and a stable baseline obtained on the chart

recorder prior to addition of the agonist. The samples were stirred continuously using magnetic stir bars and recording was continued for 6 minutes.

The extent of platelet aggregation was quantified in two ways (Figure 2.10). Firstly, by measuring the maximum height in cm of the aggregation trace after 6 minutes ( $T_{\max 6}$ ), using the baseline tracing prior to agonist addition as zero, ie no light transmission. This measurement takes no account of the rate at which platelet aggregation is occurring. This may be calculated from the slope of the aggregation trace but, in the absence of an aggregometer designed to measure this parameter automatically, it may be very inaccurate. An alternative is to measure the lag time to achieving 50% of maximal aggregation ( $LT_{50}$ , Figure 2.10), which has been shown to be a sensitive quantitative index of arachidonate-induced aggregation (298). In all the studies data for adrenaline-induced aggregation are expressed as the  $T_{\max 6}$  measuring the total height of the primary and secondary wave together. For arachidonic acid and collagen-induced aggregation the  $LT_{50}$  tended to give more sensitive and reproducible results. Normal values are given in Appendix II.

FIGURE 2.10 CALCULATION OF THE  $LT_{50}$  (LAG TIME TO ACHIEVING 50% OF MAXIMAL AGGREGATION).



### Platelet aggregation in whole blood:

Platelet aggregation in whole blood was measured by impedance aggregometry using the Chrono-log Whole Blood Aggregometer. This measures an increase in electrical impedance across electrodes placed in the blood sample as platelets accumulate on them. Venous blood collected into 1/10th volume 3.8% citrate was discharged in 1 ml aliquots into plastic cuvettes for immediate testing. After preincubation for 1-2 minutes to establish a stable baseline, the agonist was added and the resultant change in impedance monitored for 10 minutes. Collagen and arachidonic acid were used as agonists in these experiments in fixed doses of 9.5 ug/ml and 0.66mM respectively.

Results were expressed as the increase in impedance in ohms after 6 minutes. In common with some other investigators (353), I found this method less sensitive and less consistent than light transmission platelet aggregometry (see Appendix II). I therefore used the light transmission method for most of my experiments. Nevertheless, the whole blood technique offered particular advantages in assessing the effects of thromboxane synthase inhibitors and in some of these experiments both

methods were employed.

#### PLATELET GRANULE PROTEINS

Measurement of the plasma levels of the platelet-specific granule proteins,  $\beta$ -thromboglobulin and platelet factor 4, provides a useful index of the presence of platelet activation in vivo (265)(354). Interpretation of the results may be confounded by the influence of in vitro platelet activation resulting in artificially elevated values (263)(265)(355). In addition, measurement of  $\beta$ -thromboglobulin is unreliable in renal failure as the plasma concentration may rise significantly without enhanced platelet release in vivo (356). There is also considerable variation in both  $\beta$ -thromboglobulin and platelet factor 4 assays between different laboratories (357) and normal ranges must therefore be established within individual laboratories.

The problems of in vitro activation can be minimized in several ways: by careful sampling, by employing an appropriate anticoagulant containing platelet inhibitors, by rapid sample processing at 4°C and, because of their



markedly different plasma clearance rates, by measurement of both granule proteins in the same sample (265)(354). Although  $\beta$ -thromboglobulin and platelet factor 4 are released in similar quantities, the clearance of platelet factor is extremely rapid compared to that of  $\beta$ -thromboglobulin and the plasma concentration consequently significantly lower (358). Thus, comparable elevations of platelet factor 4 and  $\beta$ -thromboglobulin measured in the same sample strongly suggest in vitro platelet release. In contrast, elevated levels of  $\beta$ -thromboglobulin in the presence of normal or slightly increased levels of platelet factor 4 are consistent with in vivo platelet activation.

$\beta$ -thromboglobulin and platelet factor 4 were measured in plasma by radioimmunoassay using commercially available kits. In each case the concentration of both granule proteins was determined in the same sample. Consequently identical procedures for sample collection and preparation were followed for both assays.

## $\beta$ -Thromboglobulin

### Sample collection and preparation:

Blood was collected without stasis via a 19-gauge butterfly needle into a syringe containing an ice-cold anticoagulant mixture in a ratio of 4:1 blood:anticoagulant. The anticoagulant mixture was freshly prepared and consisted of acid citrate dextrose with 10 $\mu$ M indomethacin and 1 $\mu$ M PGE<sub>1</sub> added to minimize in vitro platelet activation. A two-syringe technique was also used to clear the system of activating factors: blood drawn into the first syringe (1-2ml) was discarded, while the second sample collected into the anticoagulant mixture was retained for analysis. The samples were kept in melted ice and centrifuged within 30 minutes of withdrawal at 30,000g at 4°C for 20 minutes. Platelet-poor plasma from the top layer was removed and stored at -20°C for later analysis.

### Radioimmunoassay procedure:

Plasma  $\beta$ -thromboglobulin was measured using the Amersham radioimmunoassay kit following the standard

procedure. The assay depends on competition between endogenous and  $I^{125}$ -labelled B-thromboglobulin for a limited number of binding sites on a rabbit  $\beta$ -thromboglobulin-specific antibody. Plasma (50ul) was incubated with  $I^{125}$ -B-thromboglobulin (200ul) and specific antibody (200ul) for 60 minutes at room temperature. After ammonium sulphate precipitation, the precipitates were counted in a gamma counter and the results calculated from a standard curve derived from concurrently run standards. Normal values ranged between 2 and 24 ng/ml with a mean of  $12.2 \pm 1$  ng/ml (see Appendix II). Elevated values of  $\beta$ -thromboglobulin were excluded from analysis if increased levels of platelet factor 4 were obtained in the same sample. Measurement of both granule proteins in samples from 38 patients identified values of platelet factor 4 in excess of 18 ng/ml as likely to be accounted for largely by in vitro platelet activation (see Appendix II).

#### Platelet Factor 4

Platelet factor 4 was measured using a radioimmunoassay kit from Abbott Laboratories following the standard procedure. Sample preparation was identical to that for

$\beta$ -thromboglobulin.  $I^{125}$ -labelled platelet factor 4 (250ul) and goat platelet factor 4 antiserum (250ul) were added to plasma samples and standards (50ul) and incubated at room temperature for 2 hours. After ammonium sulphate precipitation, the net radioactivity in the precipitate was determined in a gamma counter and the results calculated from a standard curve derived from concurrently run standards.

Normal values in healthy subjects ranged from 2-18 ng/ml with a mean of  $7.7 \pm 0.7$  ng/ml (see Appendix II). Values in excess of 18 ng/ml in healthy subjects were invariably associated with a marked increase in  $\beta$ -thromboglobulin in the same sample suggesting that platelet release had occurred in vitro during sample collection or preparation. Repeat samples on these subjects always yielded results within the normal range which I had established. I therefore excluded all samples in which platelet factor 4 levels were greater than 18 ng/ml (see Appendix II), on the assumption that significant platelet release had occurred in vitro, thus increasing the likelihood that measured increases in  $\beta$ -thromboglobulin could be attributed to platelet activation in vivo.

## CIRCULATING PLATELET AGGREGATES

The circulating platelet aggregate ratio (CPA) was measured according to the method of Wu and Hoak (350). Venous blood was collected via a 19 gauge butterfly needle without stasis. Blood samples (0.5 ml) were drawn directly into syringes containing either 2ml of buffered EDTA solution or 2ml of EDTA-formalin solution. The buffered EDTA solution was prepared by adding 3ml 0.077M EDTA to 5ml phosphate-buffered saline and 10ml distilled water; 5ml 4% formalin was added to the EDTA-formalin solution to a final volume of 20ml. Duplicate samples were taken on each occasion to control for in vitro platelet activation. The samples were gently mixed and decanted into siliconized tubes, then left to stand at room temperature for 15 minutes and centrifuged at 150g for 5 minutes to obtain platelet-rich plasma (PRP). Platelet counts in the PRP from each sample were carried out using a Coulter counter.

The CPA ratio was calculated as follows: counts in EDTA/counts in EDTA-formalin (data are therefore expressed as the inverse of that originally described by Wu and Hoak). The method is based on the principle that platelet

aggregates will be fixed in the EDTA-formalin solution and will therefore sediment to the bottom of the tube with centrifugation. The presence of circulating platelet aggregates is then reflected by a lower count in the PRP from the EDTA-formalin sample and hence an elevated CPA ratio. The CPA ratio in healthy subjects ranged from 0.812-1.116 with a mean of  $1.011 \pm 0.016$  (see Appendix II).

#### ASSAYS OF DRUGS AND THEIR METABOLITES

##### ASPIRIN AND SALICYLATE:

The experiments using very low doses of aspirin (20mg or less) required a highly sensitive assay to detect plasma levels of aspirin and salicylate with accuracy. Methods using high performance liquid chromatography lack the sensitivity and specificity to characterize aspirin kinetics at doses below 100mg (358)(359). I therefore used a stable isotope dilution assay employing gas chromatography/mass spectrometry which had a detection

limit of 10 ng/ml. This method was developed by Pedersen and FitzGerald in the laboratory in which I carried out this work and is described in detail elsewhere (360). Heptadeuterated derivatives synthesized in the laboratory were used as internal standards.

#### Sample Preparation:

Venous blood was collected into heparin with sodium fluoride (to prevent deacetylation) and immediately centrifuged at 2,000g at 4°C. The plasma was removed and stored at -20°C for later analysis.

#### Analytical Procedure:

Aliquots of plasma (300ul) were mixed with 5ml 0.06M pH 6.5 phosphate buffer containing 0.1M tetrahexylammonium hydroxide and 5ml dichloromethane containing 25ul benzylbromide. Internal standard solution containing aspirin and salicylic acid-heptadeutero benzyl esters (5ul) was then added to each sample and the samples were shaken in a mechanical shaker for 10 minutes. Following extraction and centrifugation at 2,000g for 10 minutes, the upper layer and colloid interface were removed and the organic phase evaporated to dryness under nitrogen. The sample was partitioned between hexane (2ml) and water (2ml) and the hexane phase transferred to a tube containing 1-2g sodium sulphate, shaken to mix and

decanted. After evaporation to dryness under nitrogen, the residue was dissolved in ethyl acetate (50ul) prior to gas chromatography.

#### Gas Chromatography/Mass Spectrometry:

Quantitation was accomplished with a Hewlett Packard 5980 instrument operating in the electron impact mode. Selective ion monitoring was carried out at m/z 228 for endogenous aspirin and salicylic acid and m/z 235 for the heptadeuterated internal standards. Using this method aspirin and salicylic acid could be detected in plasma after oral administration of a single 20mg dose of aspirin.

#### 3-1H-imidazol-1-yl-methyl-2-methyl-1H-indole-1-propanoic Acid (UK 38,485):

In order to confirm drug absorption, plasma concentrations of UK 38,485 were determined in all the subjects studied. These analyses were kindly undertaken by Dr P. Gibson of Chapel Laboratories (Hythe, Kent) by arrangement with Dr J.F. Faulkner, Pfizer Central Research, Groton, Connecticut. The method employed high performance liquid chromatography. Briefly, UK 38,485 was



isolated from plasma by precipitation with acetonitrile. The sample was then centrifuged at 2,000g for 5 minutes, evaporated to dryness under nitrogen and resuspended in tetramethylethylenediamine/citrate buffer. Quantitation was accomplished using a Spherisorb Phenyl column with a mobile phase of 75% methanol:25% tetramethylethylenediamine/citrate buffer. UK 38,485 and the internal standard (UK 37,418) were detected by fluorescence at an excitation wavelength of 220nm and emission of 370nm. The limit of detection of the assay was 10ng/ml.

Imidazo(1,5-2)pyridine-5-hexanoic Acid (CGS 13080):

Drug absorption was confirmed for CGS 13080 by measuring plasma levels in all the patients studied. The analyses were carried out by Dr Chan of the Ciba-Geigy Corporation, Summit, New Jersey. Plasma CGS 13080 was determined by high performance liquid chromatography. After purification using an Amberlite CG-50 cation exchange column, the samples were applied to a Cl8 u-Bondpack column with a mobile phase of 0.01M sodium-1-pentanesulphonate, pH 3, containing 27% acetonitrile. Levels of CGS 13080 and an internal standard (CGS 13067) were measured by fluorescence at an excitation

wavelength of 280nm. The limit of detection of the assay was 10 ng/ml.

#### IV. STATISTICAL METHODS

All the data in these studies were analysed by non-parametric methods, thereby avoiding assumptions as to the distribution of the variables under investigation (361). Analysis of variance was carried out either by the method of Friedman or by that of Kruskal and Wallis according to the form of the data. Subsequent pairwise comparisons were then performed by the Wilcoxon Matched-Pairs Signed-Ranks Test or the Mann-Whitney U Test as appropriate. The Lord's U Test was occasionally used where small sample size precluded other tests. The data are expressed throughout this work as the mean values  $\pm$  the standard error of the mean (SEM) except where otherwise stated.

## Chapter 3

### SELECTIVE INHIBITION OF ENDOGENOUS THROMBOXANE

#### FORMATION: Studies in Healthy Subjects

## INTRODUCTION

### I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE:

#### Study Design

Effect on platelet thromboxane formation ex vivo

Effect on thromboxane biosynthesis

Effect on prostacyclin biosynthesis

Effects on platelet function

Plasma drug levels

Discussion

### II. CYCLOOXYGENASE INHIBITION BY LOW-DOSE ASPIRIN:

Effect on thromboxane synthesis in vivo and ex vivo

Effect on prostacyclin biosynthesis

Effects on platelet function

Discussion

### III. COMBINED INHIBITION OF PLATELET CYCLOOXYGENASE AND THROMBOXANE SYNTHASE:

Effects on thromboxane formation in vivo and ex vivo

Effect on prostacyclin biosynthesis

Effects on platelet function

Discussion

#### IV. INHIBITION OF THROMBOXANE SYNTHESIS IN VIVO AND EX

VIVO: Evidence that the Relationship is Non-linear.

Study Design

Results

Discussion

#### V. PRESYSTEMIC ACETYLATION OF PLATELET CYCLOOXYGENASE:

Design of the model

Effects on platelet thromboxane formation

Effect on platelet function

Evidence for presystemic site of action-

plasma aspirin and salicylate levels

effect on prostacyclin biosynthesis

recovery of platelet thromboxane synthesis

Discussion

CONCLUSION

## Introduction

Recognition of the potent biological properties of thromboxane  $A_2$  and of the role of platelets in vascular occlusion has stimulated research into the development of therapeutic regimens designed to inhibit thromboxane-dependent platelet activation. Although several different approaches have been investigated in animal and clinical studies, the lack of a suitable assay to quantify endogenous thromboxane production has made interpretation of these studies very difficult. The principal pharmacological approaches to the inhibition of thromboxane biosynthesis are: selective blockade of thromboxane synthase and inhibition of cyclooxygenase by aspirin. The following studies utilize the newly-modified assay described in chapter 2 for 2,3-dinor thromboxane  $B_2$  (Tx-M) to evaluate the effects of these drugs on endogenous thromboxane formation in healthy human subjects.

The synthesis of specific inhibitors of thromboxane synthase represented a theoretically attractive approach. These drugs not only block platelet microsomal synthesis

of thromboxane from prostaglandin endoperoxides (274)(275)(276)(303), but also, in the presence of a source of prostacyclin synthase, enhance the generation of prostacyclin in vitro (62)(309). In diverse animal models of acute thrombosis (205)(211)(228) and other potentially thromboxane-mediated conditions (212)(216)(250)(251) the results have been promising. However, preliminary clinical studies in small groups of patients with similar conditions have generally failed to confirm these findings in man. There are likely to be many reasons for this. It may relate to the characteristics of the patients studied. For example, thromboxane  $A_2$  may play an insignificant role in the pathogenesis of some of the conditions in which the efficacy of thromboxane synthase inhibitors has been investigated. Alternatively, the principal reasons for their disappointing results in the clinical setting may be attributable to the pharmacology of the drugs themselves. In the first section of this chapter the pharmacology of a newly developed selective inhibitor of thromboxane synthase, 3-(1H-imidazol-1-yl-methyl) 2-methyl-1-H-indole-1-propanoic acid (UK 38,485), is examined and the effects of thromboxane synthase inhibitors on endogenous thromboxane formation are investigated in humans for the first time.

In contrast to thromboxane synthase inhibitors, the

value of aspirin in vascular occlusion has been under investigation for over thirty years (229-232)(267-272)(362). Initial enthusiasm for this approach was tempered by the failure of all the major clinical trials of aspirin in the secondary prevention of myocardial infarction to produce statistically significant reductions in coronary mortality. In addition, inhibition of cyclooxygenase by aspirin was clearly shown to depress prostacyclin formation in vitro and in vivo (287-291), although the inhibitory effects appear to be reduced by administering lower doses (65)(288)(290)(291)(333).

The ability of aspirin to inhibit endogenous thromboxane production has only been investigated in one previous study (291). Data from this study suggested that doses of aspirin between 80mg and 160mg daily inhibited endogenous thromboxane production by 80-90%, while the reduction in prostacyclin synthesis was significant at doses equal to or in excess of 160mg daily. On the basis of these results, I investigated the effects of a dose of aspirin predicted to substantially depress endogenous thromboxane formation without significantly reducing prostacyclin generation (120mg). The platelet-inhibitory properties and changes in thromboxane and prostacyclin biosynthesis induced by this calculated "selective" dose of aspirin are described in the second section of the



chapter.

Another approach to achieving a selective reduction in thromboxane synthesis has been suggested by Bertele et al (363). They showed that in vitro the platelet-inhibitory effects of a thromboxane synthase inhibitor were significantly enhanced by combining it with a small amount of aspirin. The amount of aspirin used was insufficient to decrease the synthesis of the prostacyclin metabolite, 6-keto-PGF<sub>1 $\alpha$</sub> , by cultured endothelial cells. The third section of this chapter examines the effects of such a combination in vivo.

The different approaches used in these studies to achieve selective inhibition of thromboxane formation revealed a consistent discrepancy between the degree of inhibition of thromboxane B<sub>2</sub> generation in serum and of its major metabolite in urine, Tx-M. This suggests that the relationship between inhibition of thromboxane synthesis in vivo and the capacity of platelets to generate thromboxane ex vivo is not linear. Such an observation would have important implications for platelet-inhibitory therapy and is investigated further in the fourth section of the chapter.

In some clinical situations the achievement of absolute

biochemical selectivity with respect to thromboxane versus prostacyclin biosynthesis may not be of functional importance. However, in the setting of platelet activation, in which endogenous thromboxane biosynthesis might be expected to be markedly enhanced and in which prostacyclin formation is known to be increased (180), selectivity is likely to be of much greater significance. The experiments in the final section of this chapter explore a novel approach to achieving a truly selective mode of administration of aspirin by acetylation of platelet cyclooxygenase in the presystemic circulation.

## I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE

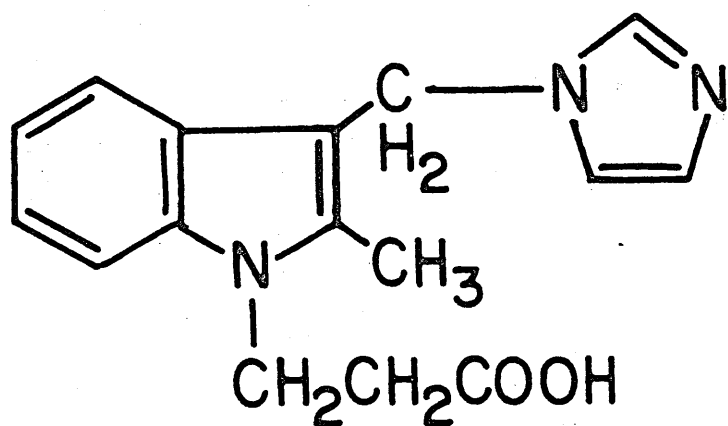
Two principal classes of thromboxane synthase inhibitors are available on a restricted basis for human studies. The majority are analogues of imidazole and include dazoxiben (UK 37,248), 3-(1H-imidazol-1-yl-methyl)-2-methyl-1-H-indole-1-propanoic acid (UK 38,485), imidazo-(1,5-2)-pyridine-5-hexanoic acid (CGS 13080) and sodium 5-(3'-pyridinylmethyl) benzofuran-2-carboxylate (U-63557A). The other class of synthase inhibitors is made up of a small number of pyridine derivatives of which the best known is sodium-(E)-3-{4-(3-pyridylmethyl)phenyl}-2-methyl-2-propenoate (OKY-1581). All of these compounds have been shown to be selective on the basis of their inhibitory activity in vitro against the various enzymes of the arachidonic acid cascade. However, their pharmacological half-lives and their potency in different animal species and types of tissue, varies for the individual compounds (277)(303)(305)(364).

An imidazole derivative, UK 38,485 (Figure 3.1), was chosen for these investigations as it had been shown in preliminary studies to have a longer biological half-life than other thromboxane synthase inhibitors available at

FIGURE 3.1 CHEMICAL FORMULA OF:-

3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid  
(UK 38,485).

UK-38,485



that time (64)(310)(364)(365)(366). It was anticipated that this might lead to enhanced platelet inhibitory properties, both by prolonging the duration of inhibition of thromboxane formation and, as a consequence of this, increasing the availability of prostaglandin endoperoxides for prostacyclin synthesis.

#### Study Design:

For the experiments described in the first three sections of this chapter, 12 healthy male volunteers aged 21-46 years were studied on 5 separate occasions. This allowed me to evaluate the effects of inhibition of thromboxane synthase, of cyclooxygenase and of combined enzyme inhibition under very tightly controlled conditions: the studies were placebo-controlled, randomized and double-blind. On each of the 5 study days, after an overnight fast, the volunteers received one of the following as a single dose: a) UK 38,485 100mg, b) UK 38,485 200mg, c) aspirin 120mg, d) aspirin 120mg plus UK 38,485 or e) placebo. The study days were separated by a minimum of 10 days and the volunteers had all refrained from ingestion of aspirin or aspirin-like drugs for at least 2 weeks prior to these investigations. UK 38,485 and placebo capsules were kindly provided by Dr P. Urquilla of

Pfizer Incorporated (Groton, Connecticut). The results of selective inhibition of thromboxane synthase by UK 38,485 are described below; those for low-dose aspirin and the aspirin/UK 38,485 combination are described in sections II and III respectively.

Effect of UK 38,485 on Platelet Thromboxane Formation ex vivo:

Platelet thromboxane generation ex vivo was measured as serum thromboxane  $B_2$  prior to and 1,4,6,8 and 24 hours after drug administration. Serum thromboxane  $B_2$  was unaffected by placebo (Figure 3.2). UK 38,485 in both doses caused a rapid, reversible reduction in serum thromboxane  $B_2$  generation which was maximal one hour after dosing (Figure 3.2). Serum thromboxane  $B_2$  fell by 96 +/- 2% from 312 +/- 33 ng/ml to 12 +/- 3 ng/ml one hour after 200mg UK 38,485. The corresponding reduction after the 100mg dose was not significantly different, falling by 96 +/- 1% from 315 +/- 26 ng/ml to 15 +/- 5 ng/ml. Serum thromboxane  $B_2$  generation remained at less than 10% of pre-dosing levels for 6 hours after UK 38,485 200mg but had returned to over 70% of pre-dosing values within 24 hours. Although at each

FIGURE 3.2 EFFECT OF UK 38,485 ON SERUM THROMBOXANE B<sub>2</sub> GENERATION IN HEALTHY SUBJECTS.

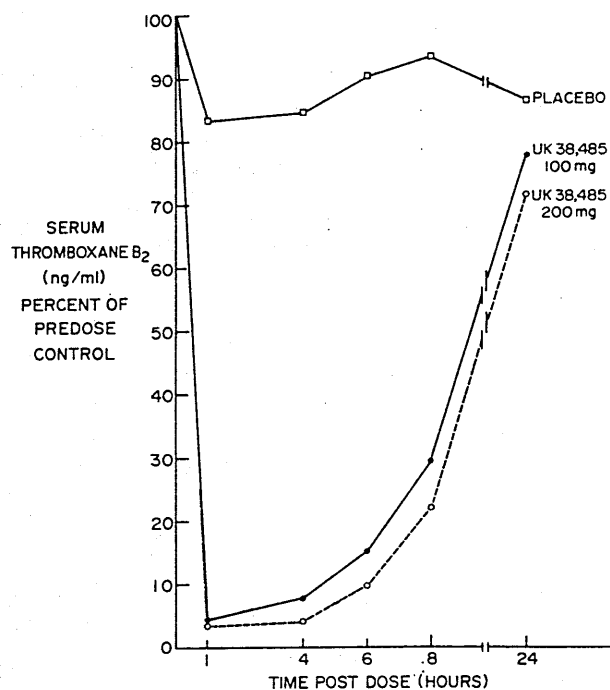
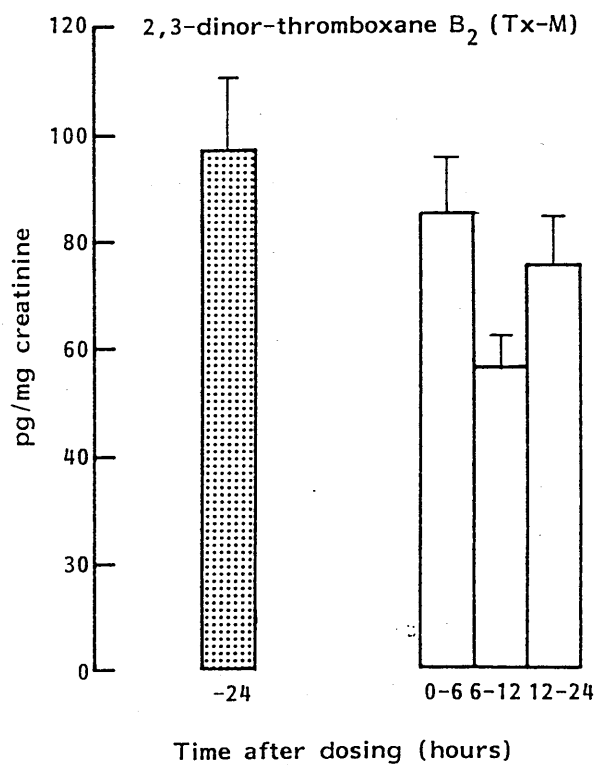


FIGURE 3.3 EFFECT OF UK 38,485 ON Tx-M EXCRETION IN HEALTHY SUBJECTS.



sampling point the degree of inhibition of serum thromboxane was slightly greater for the 200mg compared to the 100mg dose, at no time did this difference reach statistical significance.

#### Effect on Thromboxane Biosynthesis:

Endogenous thromboxane formation was determined by measuring the urinary excretion of a major urinary metabolite of thromboxane, 2,3-dinor-thromboxane B<sub>2</sub> (Tx-M), as described in Chapter 2. Urine was collected for 24 hours and in three post-dosing aliquots: 0-6, 6-12 and 12-24 hours. As there was no difference in platelet thromboxane formation ex vivo between the two doses of UK 38,485, Tx-M excretion after the 100mg dose was not assessed. There was a significant and reversible fall in Tx-M from 97 +/- 14 pg/mg creatinine to 56 +/- 6 pg/mg creatinine (p<0.01) 6-12 hours after administration of UK 38,485 (Figure 3.3). This represented a peak reduction of 37 +/- 9% from control values and contrasts with the peak decrease in serum thromboxane of 96%.



### Effects on Prostacyclin Biosynthesis:

Prostacyclin biosynthesis was determined by measuring its major metabolite in urine, 2,3-dinor-6-keto-PGF<sub>1 $\alpha$</sub>  (PGI-M), as described in Chapter 2. PGI-M was assayed in the same aliquots of urine collected for Tx-M estimation, ie. for 24 hours before and 0-6, 6-12 and 12-24 hours after drug administration. The results are shown in Table 3.1. There was a transient but significant ( $p < 0.05$ ) increase in PGI-M excretion after the higher dose of UK 38,485 which was maximal 6-12 hours after dosing and corresponded to the time of peak Tx-M depression. PGI-M increased by 20% from control values from  $119 \pm 18$  pg/mg creatinine to  $143 \pm 19$  pg/mg creatinine. No significant increase in PGI-M was apparent after 100mg UK 38,485.

TABLE 3.1 EXCRETION OF 2,3-DINOR-6-KETO-PGF<sub>1 $\alpha$</sub>  (PGI-M) IN 12 HEALTHLY MALE SUBJECTS AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485 (100mg and 200mg) OR PLACEBO.

Time after dosing (hours)	Placebo	UK 38,485 100mg	UK 38,485 200mg
-24	111 $\pm$ 18	120 $\pm$ 17	119 $\pm$ 18
0-6	115 $\pm$ 16	126 $\pm$ 24	122 $\pm$ 16
6-12	124 $\pm$ 14	131 $\pm$ 24	143 $\pm$ 19*
12-24	128 $\pm$ 15	126 $\pm$ 16	131 $\pm$ 19

Values are expressed in pg/mg creatinine (mean  $\pm$  SEM).

\*  $p < 0.05$  compared to placebo

### Effects on Platelet Function:

The effects on platelet function were assessed by measuring platelet aggregation in platelet-rich plasma (PRP) prior to and 1,4,6,8 and 24 hours after administration of placebo or UK 38,485. Arachidonic acid, collagen and adrenaline were used as agonists. In 6 patients whole blood aggregation in response to arachidonic acid and collagen was also tested. As a measure of platelet function in vivo, template bleeding times were performed prior to and 1 hour after dosing (this time point being chosen to correspond with peak inhibition of platelet thromboxane generation ex vivo).

UK 38,485 caused significant inhibition of platelet aggregation in PRP in response to 0.66mM and 1.33mM arachidonic acid for up to 8 hours following drug administration (Tables 3.2 and 3.3). Inhibition of aggregation was measured as a prolongation of the  $LT_{50}$  (lag time to achieving 50% of maximal aggregation), which has been shown to be a sensitive quantitative index of inhibition of arachidonate-induced aggregation (298)(367). There was no statistically

**TABLE 3.2 PLATELET AGGREGATION IN PRP IN RESPONSE TO ARACHIDONIC ACID (1.33mM) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.**

Time after dosing (hours)	LT <sub>50</sub> (minutes)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	0.70±0.07	0.74±0.06	0.80±0.09
1	0.68±0.04	2.10±0.68*	2.10±0.68**
4	0.66±0.05	1.75±0.59*	2.53±0.74**
6	0.61±0.03	1.43±0.44*	1.51±0.42**
8	0.61±0.03	1.37±0.45*	1.57±0.52**
24	0.67±0.03	1.12±0.45	1.34±0.44

**TABLE 3.3 PLATELET AGGREGATION IN PRP IN RESPONSE TO ARACHIDONIC ACID (0.66mM) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.**

Time after dosing (hours)	LT <sub>50</sub> (minutes)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	0.97±0.13	0.98±0.11	0.91±0.07
1	0.90±0.14	2.67±0.72**	2.80±0.70**
4	0.82±0.11	2.59±0.73*	2.34±0.66*
6	0.80±0.06	2.62±0.73*	1.59±0.43*
8	0.91±0.11	1.85±0.58	2.02±0.57*
24	0.94±0.09	1.36±0.45	1.90±0.57

Values are mean ± SEM of determinations in 12 healthy male subjects.

LT<sub>50</sub> : lag time to achieving 50% of maximal aggregation.

\* p <0.05 vs placebo

\*\* p <0.01 vs placebo

significant difference between the two doses of UK 38,485 in the degree of inhibition observed. Platelet aggregation induced by high dose collagen (19 ug/ml) was also reversibly inhibited by both doses of UK 38,485 as shown in Table 3.4. The degree of inhibition was minor and considerably less than that seen in response to arachidonic acid, reaching statistical significance only at 4 and 6 hours after drug administration. The 200mg dose of UK 38,485 caused transient inhibition of aggregation in response to low-dose collagen (Table 3.5). This effect was not apparent after the 100mg dose of UK 38,485. Platelet aggregation induced by adrenaline (1uM, 5uM and 10uM) was unaffected by either dose of UK 38,485 (Tables 3.6, 3.7 and 3.8).

The effects of UK 38,485 on platelet aggregation in whole blood were investigated in 6 subjects. Although the extent of aggregation (measured as the height of the aggregation trace in cm above the baseline after 6 minutes) was reduced in response to 0.66mM arachidonic acid, the difference failed to reach statistical significance (Table 3.9). There was also no significant inhibition of collagen-induced aggregation in whole blood (Table 3.10). The effect of UK 38,485 on the template bleeding time is shown in Figure 3.4. After the lower dose

TABLE 3.4 PLATELET AGGREGATION IN PRP INDUCED BY COLLAGEN (19 $\mu$ g/ml) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	LT <sub>50</sub> (minutes)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	1.98 $\pm$ 0.09	2.18 $\pm$ 0.12	2.10 $\pm$ 0.11
1	2.0 $\pm$ 0.09	2.28 $\pm$ 0.17	2.33 $\pm$ 0.14
4	1.99 $\pm$ 0.09	2.38 $\pm$ 0.13*	2.84 $\pm$ 0.14**
6	2.0 $\pm$ 0.09	2.25 $\pm$ 0.12	2.41 $\pm$ 0.14**
8	2.0 $\pm$ 0.11	2.11 $\pm$ 0.13	2.12 $\pm$ 0.12
24	2.22 $\pm$ 0.09	2.08 $\pm$ 0.09	2.28 $\pm$ 0.14

Values are mean  $\pm$  SEM of determinations in 12 healthy male subjects.

LT<sub>50</sub> : lag time to achieving 50% of maximal aggregation.

TABLE 3.5 PLATELET AGGREGATION IN PRP INDUCED BY COLLAGEN (1.9 $\mu$ g/ml) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	LT <sub>50</sub> (minutes)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	4.44 $\pm$ 0.24	4.96 $\pm$ 0.31	5.04 $\pm$ 0.29
1	4.64 $\pm$ 0.24	4.98 $\pm$ 0.33	4.82 $\pm$ 0.19
4	4.54 $\pm$ 0.27	5.23 $\pm$ 0.34	5.34 $\pm$ 0.25
6	4.64 $\pm$ 0.19	5.19 $\pm$ 0.30	3.45 $\pm$ 0.23*
8	4.54 $\pm$ 0.21	4.63 $\pm$ 0.36	5.13 $\pm$ 0.25
24	4.60 $\pm$ 0.15	5.36 $\pm$ 0.17	5.30 $\pm$ 0.20

Values are mean  $\pm$  SEM of determinations in 11 healthy male subjects. (one subject consistently failed to respond to low dose collagen).

LT<sub>50</sub> : lag time to achieving 50% of maximal aggregation.

\* p < 0.05 vs placebo

TABLE 3.6 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE (10 $\mu$ M) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485

Time after dosing (hours)	Tmax <sub>6</sub> (cm)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	16.1 $\pm$ 0.8	16.4 $\pm$ 0.7	16.6 $\pm$ 0.6
1	17.0 $\pm$ 0.5	16.0 $\pm$ 0.7	17.0 $\pm$ 0.4
4	17.3 $\pm$ 0.6	17.1 $\pm$ 0.4	16.4 $\pm$ 0.4
6	17.8 $\pm$ 0.6	15.9 $\pm$ 0.5	17.1 $\pm$ 0.5
8	17.8 $\pm$ 0.7	16.7 $\pm$ 0.7	18.2 $\pm$ 0.6
24	17.0 $\pm$ 0.5	16.8 $\pm$ 0.8	17.9 $\pm$ 0.6

TABLE 3.7 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE (5 $\mu$ M) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	Tmax <sub>6</sub> (cm)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	16.5 $\pm$ 0.5	16.9 $\pm$ 0.6	15.7 $\pm$ 1.4
1	16.5 $\pm$ 0.4	16.4 $\pm$ 0.3	14.9 $\pm$ 1.0
4	16.8 $\pm$ 0.4	17.0 $\pm$ 0.5	15.7 $\pm$ 0.8
6	16.8 $\pm$ 0.6	15.9 $\pm$ 0.6	15.3 $\pm$ 0.8
8	17.2 $\pm$ 0.5	16.8 $\pm$ 0.6	14.0 $\pm$ 1.5
24	17.9 $\pm$ 0.5	16.7 $\pm$ 0.5	16.8 $\pm$ 1.2

Values are mean  $\pm$  SEM of determinations in 12 healthy male subjects.

Tmax<sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist.

TABLE 3.8 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE (1 $\mu$ M) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	Tmax <sub>6</sub> (cm)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	16.5 $\pm$ 0.5	14.2 $\pm$ 0.9	15.1 $\pm$ 1.0
1	16.6 $\pm$ 0.6	15.7 $\pm$ 0.5	16.5 $\pm$ 0.6
4	16.4 $\pm$ 0.5	14.9 $\pm$ 2.0	14.4 $\pm$ 2.3
6	16.6 $\pm$ 0.7	16.1 $\pm$ 0.4	16.5 $\pm$ 0.6
8	18.6 $\pm$ 0.9	16.0 $\pm$ 0.4	16.3 $\pm$ 2.0
24	16.4 $\pm$ 0.7	17.1 $\pm$ 0.7	17.7 $\pm$ 0.7

Values are mean  $\pm$  SEM of determinations in 6 healthy male subjects.

Tmax<sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist

TABLE 3.9 PLATELET AGGREGATION IN WHOLE BLOOD IN RESPONSE TO ARACHIDONIC ACID (0.66mM) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	Change in impedance (ohms)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	14.5±1.1	10.9±1.2	10.7±1.2
1	14.5±2.8	9.2±3.0	8.1±0.8
4	14.1±1.7	9.9±2.1	6.7±1.1
6	12.1±2.1	10.3±2.1	9.3±0.7
8	13.0±1.0	12.7±1.8	10.7±1.1

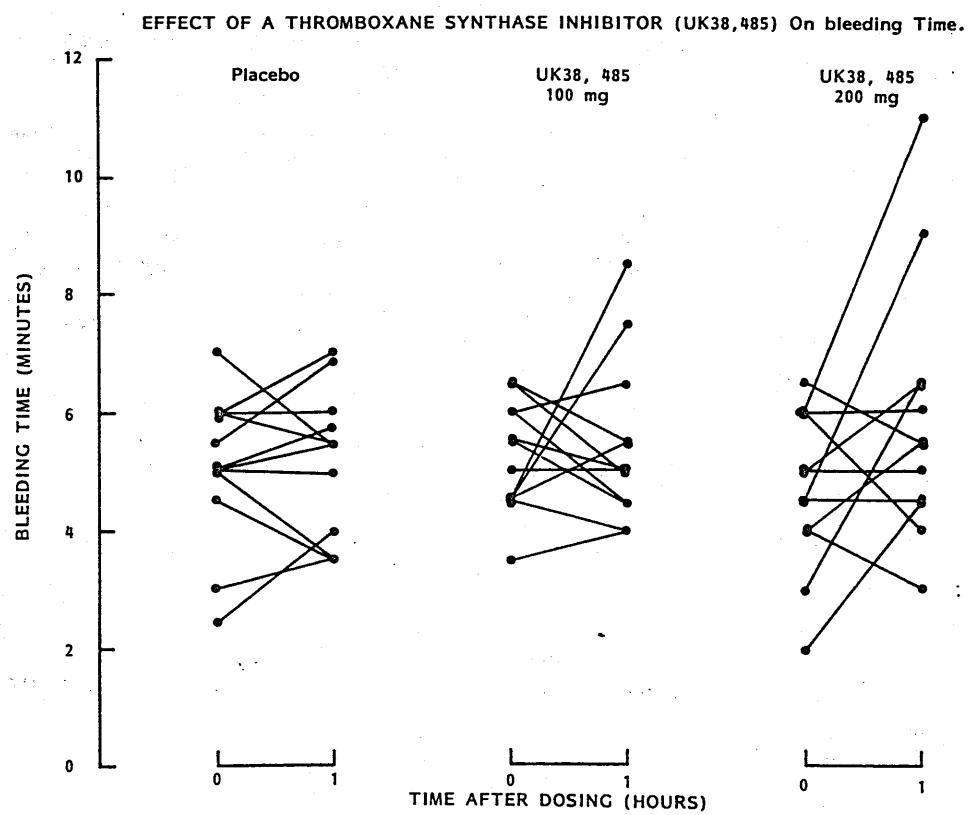
TABLE 3.10 PLATELET AGGREGATION IN WHOLE BLOOD IN RESPONSE TO COLLAGEN (9.5µg/ml) AFTER SINGLE DOSE ADMINISTRATION OF UK 38,485.

Time after dosing (hours)	Change in impedance (ohms)		
	Placebo	UK 38,485 100mg	UK 38,485 200mg
0	12.4±1.4	10.6±0.9	10.6±0.9
1	11.6±1.2	7.4±0.8	7.7±1.7
4	11.9±0.7	10.2±1.0	9.7±0.9
6	10.8±0.8	9.9±0.5	10.3±1.1
8	10.9±0.8	10.1±1.3	9.7±0.7

Values are mean ± SEM of determinations in 6 healthy male subjects.

There was no significant difference between placebo and UK 38,485 by analysis of variance before and after drug administration.

FIGURE 3.4





the bleeding time was slightly prolonged from 5.1 +/- 0.3 to 5.4 +/- 0.4 minutes and after the 200mg dose from 4.7 +/- 0.4 to 5.9 +/- 0.6 minutes. Although there was a 2-fold increase in bleeding time after the higher dose in 4 of the subjects, in the group as a whole the increment was not statistically significant.

#### Plasma Drug Levels:

Plasma levels of UK 38,485 were obtained 1,4,6 and 8 hours after drug administration. The results are shown in Table 3.11. Plasma concentrations of UK 38,485 were proportional to dose. Peak drug levels were measured one hour after dosing: 200mg-: 3207 +/- 333 ng/ml (range 1954-4520) and 100mg-: 1699 +/- 140 ng/ml (range 768-2670 ng/ml). The decline in plasma levels of UK 38,485 was rapid, less than 3% of the peak drug concentration being detectable after 6 hours. Plasma concentrations of UK 38,485 in the patient in whom only very minor inhibition of platelet aggregation was observed were similar to those for the whole group for each dose of UK 38,485 and at each time point.

TABLE 3.11 PLASMA CONCENTRATIONS OF UK 38,485 (ng/ml)

Time after dosing (hours)	UK 38,485 100mg	UK 38,485 200mg
0	ND	ND
1	1699±140	3207±333
4	99±13	225±46
6	38±11	66±8
8	16±8	26±4

Values are expressed as mean ± SEM in 12 healthy male subjects.

Limit of sensitivity : 10ng/ml.

ND= Not detectable.

## Discussion

A newly developed thromboxane synthase inhibitor, UK 38,485, has been shown in this study to selectively and reversibly inhibit endogenous thromboxane formation in normal subjects. Despite approaching maximal inhibition of the capacity of platelets from these individuals to synthesize thromboxane  $A_2$ , the effects on platelet function were transient and relatively minor. Thus, while there was a slight increase in the bleeding time, this did not reach statistical significance. Similarly, although there was significant inhibition of arachidonic acid-induced platelet aggregation consistent with the reduction in platelet thromboxane generation, the responses to adrenaline and collagen were maintained. There was also no alteration in platelet aggregation in whole blood. This is consistent with the relatively small increase caused by UK 38,485 in endogenous prostacyclin synthesis, which was several orders of magnitude less than would result in systemically active concentrations of prostacyclin in the circulation (132)(155).

The effects of other thromboxane synthase inhibitors on

human platelet behaviour have been investigated by several groups and shown to be minor in comparison to those of aspirin. Most have demonstrated similar dose-related inhibition of arachidonate and collagen-induced aggregation after administration either of other imidazole-analogue synthase inhibitors, such as dazoxiben (64)(65)(309)(368)(369)(370), or of pyridine derivatives, for example, sodium (E)-3-(4-{3-pyridylmethyl}phenyl)-2-methyl-2-propenoate (OKY 1581) (310)(371). The effects of thromboxane synthase inhibition on the bleeding time have been variable. Lower doses have no effect (64)(308). Higher doses have been shown in some studies (64)(65)(369), but not in others (310)(371), to significantly prolong the bleeding time by up to 100%.

The discrepancy between the marked inhibition of platelet thromboxane formation *ex vivo* and the relatively weak platelet inhibitory properties has important clinical implications and may have several explanations. Firstly, accumulated prostaglandin endoperoxides may reach sufficient concentrations, when their metabolism via thromboxane synthase is blocked, to substitute for the proaggregatory effects of thromboxane  $A_2$ . The lack of effect on adrenaline- and low dose collagen-induced aggregation, which are largely prostaglandin-dependent, is consistent with this hypothesis. In addition, experiments

in vitro show that while platelets incubated with the thromboxane synthase inhibitor dazoxiben continue to aggregate in response to arachidonic acid despite virtually complete inhibition of thromboxane formation, the addition of small amounts of aspirin abolishes aggregation (363). Nevertheless, direct evidence of endoperoxide-mediated platelet aggregation in this setting is lacking in humans at present. Recent studies in a canine model of coronary artery occlusion, however, do support the concept of endoperoxide-dependent platelet activation (345). In this model, Fitzgerald et al showed that the combination of an endoperoxide/thromboxane  $A_2$  receptor antagonist with a synthase inhibitor, but not the synthase inhibitor alone, significantly delayed the time taken for complete coronary occlusion to occur. Preliminary evidence for the efficacy of this combination has also been obtained with human platelets in vitro (372)(373).

An alternative explanation for the weak platelet inhibitory activity seen after administration of thromboxane synthase inhibitors may be provided by comparing the effects of these drugs on the platelet capacity to generate thromboxane ex vivo (measured as serum thromboxane  $B_2$ ) and their effects on actual thromboxane synthesis in vivo. This study investigated for

the first time the effects of acute administration of a synthase inhibitor on thromboxane formation in vivo by measuring urinary excretion of the thromboxane metabolite, Tx-M. Interestingly, although the peak reduction in serum thromboxane generation ex vivo exceeded 95%, the mean maximal decrease in Tx-M, reflecting thromboxane synthesis in vivo, was less than 40%. Thus, incomplete inhibition of thromboxane biosynthesis may have contributed to the weak anti-platelet effects of UK 38,485. This observation was further investigated in studies described in this and subsequent chapters.

Several investigators have reported interindividual differences in the platelet inhibitory response to thromboxane synthase inhibitors in vitro (307)(374). This has led to the suggestion that subjects may be divided into two distinct groups on the basis of their in vitro platelet response to these drugs: "responders" and "non-responders". Experiments in vitro have demonstrated a relationship between "responder" status and the ability to produce  $\text{PGD}_2$  (375)(376)(377). Measures which increase the generation of  $\text{PGD}_2$  induce "non-responder" platelets to respond. Thus, it has been proposed that the anti-platelet effects of thromboxane synthase inhibitors are dependent upon inducing a  $\text{PGD}_2$ -mediated rise in intraplatelet cyclic AMP and

that interindividual variation in this response explains the inconstant effects of these drugs on platelet function. However, the proportion of "non-responders" varies considerably in different studies, from around 33% (370)(378) up to 75% (375)(376) and there is little evidence of a clear division between "responders" and "non-responders" in vivo.

In my study, by using a sensitive measure of inhibition of platelet aggregation, the  $LT_{50}$  (298)(367), which takes account of both the rate and extent of aggregation, I was able to demonstrate an impaired aggregation response in all 12 subjects after administration of the higher dose of UK 38,485. Similarly, in experiments I carried out with others in our laboratory, we showed that by prolonging the incubation time of platelet suspensions with a thromboxane synthase inhibitor (dazoxiben) in vitro, it was possible to progressively delay the onset of platelet aggregation and ultimately completely inhibit aggregation (379). In further experiments, we found that this was accompanied by further inhibition of platelet thromboxane generation and conversion of apparent "non-responders" (in vitro) into "responders". Thus, "responder" status would not be expected to be of major importance in determining the anti-platelet effects of thromboxane synthase inhibitors in vivo.

The principal theoretical advantage of thromboxane synthase inhibitors over alternative approaches to the inhibition of thromboxane biosynthesis is their capacity to increase prostacyclin formation. There is evidence from animal studies that such a mechanism is relevant in vivo. In a canine model of partial circumflex coronary obstruction, while a synthase inhibitor prevented blockage of the artery in 90% of dogs, this effect was inhibited by topical application of a prostacyclin synthase inhibitor (203). In a similar model, the combination of a thromboxane synthase inhibitor with an endoperoxide/thromboxane  $A_2$  receptor antagonist was more effective in preventing coronary artery occlusion than the receptor antagonist alone (345). Thus, prostacyclin appears to contribute to the efficacy of thromboxane synthase inhibitors in this setting.

The mechanisms by which these drugs enhance prostacyclin synthesis are not fully understood. Since it has been reported to occur in vivo with synthase inhibitors which are structurally distinct from UK 38,485 (64)(65), it is unlikely to be the result of a direct stimulatory effect of the drug itself. A more attractive hypothesis is that the increment in prostacyclin generation is a consequence of redirection of



prostaglandin endoperoxides to a source of prostacyclin synthase. This might occur in any tissue with the enzymes to synthesize both thromboxane and prostacyclin, such as the lung. Alternatively, platelet-derived endoperoxides might be utilized by endothelial cells to synthesize prostacyclin at sites of platelet-vessel wall interactions. There is good evidence that this takes place in vitro. Marcus et al clearly showed that aspirin-treated endothelial cells are able to synthesize prostacyclin from endoperoxides derived from platelets and that synthesis is enhanced in the presence of a thromboxane synthase inhibitor (62). Others have confirmed these findings (63), but direct evidence of such a mechanism occurring in vivo remains to be established in humans.

The biochemical and functional effects of a novel thromboxane synthase inhibitor have been described and discussed. In common with other synthase inhibitors, the effects on platelet function are relatively minor despite over 95% inhibition of thromboxane generation ex vivo. The results suggest that this is likely to be due in part to the accumulation of proaggregatory endoperoxides and their substitution for thromboxane  $A_2$  in the presence of thromboxane synthase blockade. However, the concomitant finding of a relatively small reduction in a major urinary metabolite of thromboxane, Tx-M, raises the possibility

that incomplete inhibition of thromboxane biosynthesis may also be a contributory factor. These observations are further investigated in the following studies.

## II. CYCLOOXYGENASE INHIBITION BY LOW-DOSE ASPIRIN

The dose of aspirin used in this study, 120mg, was selected with the aim of achieving maximal inhibition of thromboxane formation without significantly reducing prostacyclin synthesis. Patrono et al showed that the relationship between the dose of aspirin administered and the resultant degree of inhibition of serum thromboxane generation was linear (297). They found a 50% inhibitory dose for single dose administration of 26mg. Thus, a dose of 180mg would be required to reduce serum thromboxane by over 99%. However, there is clear evidence that even in doses lower than this, aspirin inhibits prostacyclin synthesis by human vascular tissue in vitro (288)(289)(290) and at doses of 160mg or greater it will significantly inhibit endogenous prostacyclin biosynthesis (291). Single doses of 100mg have been shown to depress serum thromboxane  $B_2$  by around 95% (333)(336). I therefore chose a dose of 120mg in order to increase the degree of inhibition of thromboxane generation attained, while still leaving prostacyclin synthesis relatively unaltered. The effects of single dose administration of

low-dose aspirin on endogenous thromboxane biosynthesis had not been previously investigated and thus could not be allowed for in calculating the most appropriate dose of aspirin to employ.

Effects of Low-dose Aspirin on Thromboxane Formation in vivo and ex vivo:

Platelet thromboxane formation ex vivo was determined in serum before and 1,4,6,8 and 24 hours after single dose administration of 120mg of aspirin. Thromboxane synthesis in vivo was measured as Tx-M excretion in the 24 hours prior to dosing and in three aliquots for 24 hours following aspirin administration (0-6, 6-12 and 12-24 hours).

Serum thromboxane  $B_2$  fell by a mean of  $94 \pm 2$  % one hour after dosing from  $349 \pm 37$  to  $18 \pm 4$  ng/ml and remained inhibited for the 24 hour study period (Figure 3.5). Aspirin also caused a significant reduction in Tx-M excretion from  $134 \pm 21$  to  $92 \pm 17$  pg/mg creatinine ( $p < 0.01$ ) which, in contrast to the thromboxane synthase inhibitor, persisted for 24 hours after drug administration (Figure 3.5). However, peak depression of

Figure 3.5 EFFECTS OF LOW-DOSE ASPIRIN (120mg) ON SERUM THROMBOXANE B<sub>2</sub> GENERATION AND Tx-M EXCRETION IN HEALTHY SUBJECTS.

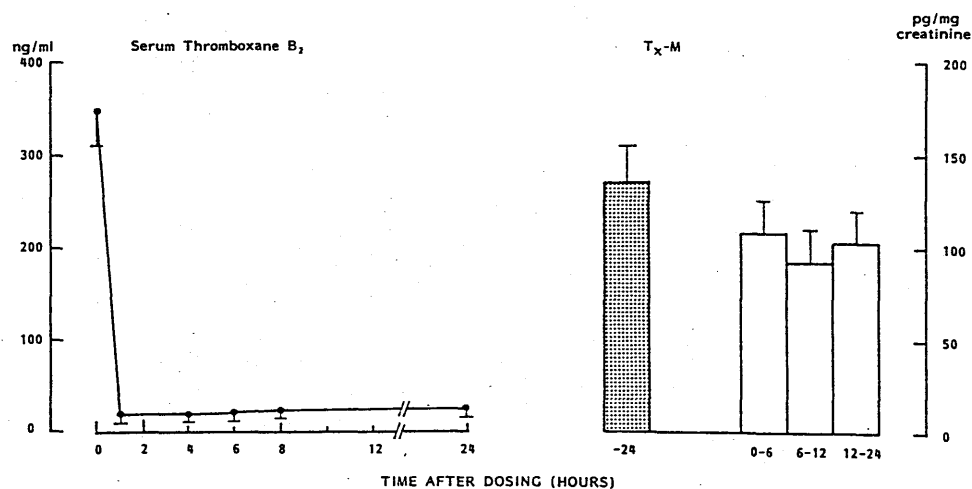


TABLE 3.12 EXCRETION OF Tx-M, SERUM THROMBOXANE B<sub>2</sub> AND PLASMA SALICYLATE AFTER SINGLE DOSE ADMINISTRATION OF SODIUM SALICYLATE 120mg.

	Prior to dosing	After dosing	
		Time (hrs)	Concentration
Plasma salicylate (µg/ml)	ND	3	7.6±1.2
Serum thromboxane B <sub>2</sub> (ng/ml)	345±75	3	313±29
Tx-M (pg/mg creatinine)	75±13	0-6	85±28
		6-12	78±10
		12-24	72±7

Values are mean ± SEM of determinations in 3 healthy male subjects.

ND = Not detectable.

Tx-M, which was observed in the 6-12 hour aliquot after dosing, was only 28 +/- 8 % from pre-dosing values despite almost maximal inhibition of serum thromboxane B<sub>2</sub> formation throughout the study period. To exclude the possibility that the change in Tx-M excretion resulted from altered renal handling of Tx-M rather than inhibition of cyclooxygenase (380), the effects of an identical oral dose (120mg) of sodium salicylate were investigated in three of the subjects. Sodium salicylate is subject to the same renal tubular excretory mechanism as aspirin (381) but has no or only very weak inhibitory activity against platelet cyclooxygenase (6)(382)(383). In contrast to aspirin, 120mg of sodium salicylate did not alter Tx-M excretion or the capacity of platelets to form thromboxane B<sub>2</sub> (Table 3.12).

#### Effect on Prostacyclin Biosynthesis:

Urinary excretion of the prostacyclin metabolite PGI-M was measured prior to dosing and in three aliquots following dosing with aspirin 120mg (0-6, 6-12 and 12-24 hours). Analysis of Tx-M and PGI-M in the same samples allowed direct comparison of the inhibitory effects of

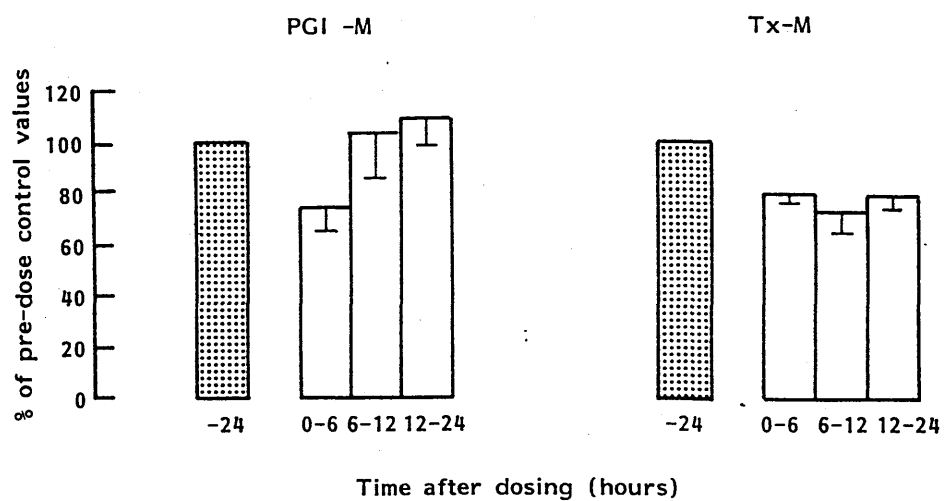
this dose of aspirin on endogenous biosynthesis of both thromboxane and prostacyclin in the 12 subjects studied.

As shown in Figure 3.6, even in this low dose aspirin caused a small but statistically significant reduction in PGI-M. The decrease in prostacyclin metabolite was, however, transient. PGI-M excretion fell from  $100 \pm 10$  to  $70 \pm 11$  pg/mg creatinine 0-6 hours after aspirin administration ( $p < 0.01$ ) and returned to normal levels in the second two aliquots collected ( $104 \pm 22$  and  $110 \pm 16$  pg/mg creatinine in the 6-12 and 12-24 hour samples respectively). The mean maximum reduction in PGI-M ( $25 \pm 11\%$ ) was similar to that for Tx-M ( $28 \pm 8\%$ ), but the inhibitory effects on Tx-M were more prolonged, no recovery being seen within the 24 hour study period (Figure 3.6).

#### Effects on Platelet Function:

Platelet aggregation in PRP in response to arachidonic acid, collagen and adrenaline was assessed before and 1,4,6,8 and 24 hours after aspirin administration. The bleeding time was measured immediately prior to dosing and

FIGURE 3.6 PERCENTAGE REDUCTION IN PGI-M AND Tx-M EXCRETION AFTER ADMINISTRATION OF LOW-DOSE ASPIRIN TO HEALTHY SUBJECTS.





one hour following 120mg aspirin. Aspirin completely abolished platelet aggregation induced by arachidonic acid and low-dose collagen throughout the 24 hour study period (Table 3.13). In addition, aspirin inhibited the secondary wave of adrenaline-induced aggregation and significantly impaired, although did not abolish, platelet aggregation in response to high-dose collagen (Table 3.13). Aspirin 120mg also caused a small but significant prolongation of the bleeding time from  $5.1 \pm 0.4$  to  $7.4 \pm 0.5$  minutes ( $p < 0.01$ ).

### Discussion

The effects of single dose administration of 120mg of aspirin on endogenous thromboxane and prostacyclin biosynthesis and platelet function have been investigated in healthy subjects. Platelet thromboxane  $B_2$  formation in serum was rapidly inhibited by 120mg of aspirin by a mean of 94%. Despite sustained inhibition of the platelet capacity for thromboxane generation ex vivo for at least 24 hours, the corresponding reduction in Tx-M, reflecting actual thromboxane synthesis in vivo, was

TABLE 3.13 EFFECT OF SINGLE DOSE ADMINISTRATION OF ASPIRIN (120mg) ON PLATELET AGGREGATION AND BLEEDING TIME.

TIME AFTER DOSING (hours)	PLATELET AGGREGATION IN PRP						TEMPLATE BLEEDING TIME (minutes)
	Arachidonic Acid* 0.66mM	Arachidonic Acid* 1.33mM	Collagen* 1.9µg/ml	Collagen* 19µg/ml	Adrenaline 1µM	Adrenaline 10µM	
0	1.32±0.3	0.83±0.1	4.46±0.3	2.09±0.1	PA+SA	PA+SA	5.1±0.4
1	NA	NA	NA	2.52±0.1 <sup>‡</sup>	PA	PA	7.4±0.5 <sup>‡</sup>
4	NA	NA	NA	2.59±0.2 <sup>‡</sup>	PA	PA	not tested
6	NA	NA	NA	2.53±0.1 <sup>‡</sup>	PA	PA	not tested
8	NA	NA	NA	2.57±0.1 <sup>‡</sup>	PA	PA	not tested
24	NA	NA	NA	2.65±0.2 <sup>‡</sup>	PA	PA	not tested

Values are mean ± SEM of determinations in 12 healthy male subjects.

\* LT<sub>50</sub> (lag time to achieving 50% of maximal aggregation, minutes); ‡ p < 0.01.

NA = No aggregation.

PA = Primary wave of aggregation.

SA = Secondary wave of aggregation.

less than 30%. The failure of sodium salicylate to alter Tx-M excretion suggests that the reduction in Tx-M was indeed due to cyclooxygenase inhibition. However two possible explanations for the significantly smaller depression in Tx-M compared to serum thromboxane should be considered.

Firstly, since Tx-M reflects total body synthesis of thromboxane, it is derived from both platelet and extraplatelet sources, for example from macrophages, the kidney and possibly the lung (25)(33)(34)(72)(77). Thus, the significant amounts of Tx-M excreted after aspirin administration may represent thromboxane synthesis from cells other than platelets on which aspirin has had less profound inhibitory effects. Previous experiments (see Chapter 2), however, suggested that in healthy subjects Tx-M is predominantly derived from platelets. In addition, patients with thrombocytosis and increased platelet turnover had markedly elevated Tx-M excretion. The pattern of recovery of inhibition of Tx-M in the present studies is also consistent with inhibition of platelet-derived Tx-M. Levels of Tx-M rapidly returned towards normal after the reversible thromboxane synthase inhibitor. In contrast, after low-dose aspirin (and after the higher dose administered in the preliminary investigations in Chapter 2), Tx-M remained inhibited for at least 24 hours.

Others have shown a similarly reversible depression in Tx-M following administration of indomethacin, a reversible cyclooxygenase inhibitor (266). It therefore seems unlikely that the discrepancy between the degree of inhibition of Tx-M and serum thromboxane is entirely accounted for by non-platelet thromboxane synthesis, although a small proportion of the residual Tx-M may originate from such sources.

An alternative explanation is that Tx-M and serum thromboxane  $B_2$  formation are not related in a linear fashion. There is some evidence to suggest that the relationship between inhibition of the platelet capacity for thromboxane generation ex vivo and thromboxane-dependent platelet activation, for example, is not linear (291)(384)(385). In the same way, the results of this study suggest that the relationship between the extent of inhibition of serum thromboxane  $B_2$  formation and of Tx-M excretion may also be non-linear. Such an observation, although not previously recognized, would not be surprising since it is well known that the capacity of tissues to synthesize eicosanoids in response to physical and chemical stimuli far exceeds their actual production rates in vivo (92)(132)(181)(288)(336).

The dose of aspirin used in this study had significant

anti-platelet activity by conventional criteria. Platelet aggregation induced by arachidonic acid, collagen and adrenaline was inhibited and the bleeding time was prolonged by almost 50%. However, a major potential disadvantage of even this low dose of aspirin is the inhibitory effect it exerted on prostacyclin biosynthesis. A single dose of 120mg depressed prostacyclin synthesis in vivo by 30%. The fall in prostacyclin was transient and is consistent with rapid de novo synthesis of endothelial cyclooxygenase following aspirin exposure as has been shown to occur in cultured endothelial cells (287)(294). The rapid recovery of PGI-M after single dose administration of aspirin contrasts with the delay of several days seen with chronic administration of much larger doses (291). This may reflect inhibition of prostacyclin synthesis by vascular smooth muscle. Chronic dosing might be expected to facilitate acetylation of vascular smooth muscle cyclooxygenase which is likely to have limited exposure to pharmacologically active concentrations of aspirin after a single low dose. Prostacyclin formation by endothelial cells appears to recover more quickly than in vascular smooth muscle cells (287)(386), perhaps due to additional inhibitory effects of aspirin on smooth muscle prostacyclin synthesis which can only be overcome by cell division (387).

The inhibitory effects of aspirin on the capacity of human venous tissue to generate prostacyclin ex vivo may be even greater for equivalent doses of aspirin than the effects on endogenous biosynthesis. Using a bioassay, Hanley et al have shown significantly reduced prostacyclin formation (around 60% inhibition) after either a single 81mg dose of aspirin (289) or after three 40mg doses administered up to 72 hours apart (388). Preston and his colleagues administered a single 300mg dose of aspirin to 4 healthy subjects and demonstrated 61-100% inhibition of venous prostacyclin synthesis ex vivo 2-8 hours after dosing (288). In a separate study, they found that the inhibitory effects persisted for at least 72 hours and that a similar degree of inhibition of prostacyclin generation could be obtained by administering four 40mg doses of aspirin 24 hours apart (389). Since these studies were carried out, Weksler and her colleagues have reported in a recent paper that even 20mg aspirin administered on a chronic basis to patients with atherosclerosis is sufficient to reduce both aortic and saphenous vein prostacyclin production by 50% (386).

In summary, it seems unlikely that a truly selective dose of aspirin administered on a daily or even alternate day basis can be identified. Chronic administration of even 20mg daily will result in some inhibition of

prostacyclin production (291)(386). The increase in aspirin dose which would be required to achieve maximal inhibition of thromboxane synthesis in vivo is likely to further compromise the biochemical selectivity of low-dose aspirin and lead to a substantial reduction in prostacyclin biosynthesis.

Although the functional implications of this are unclear at present, endogenous prostacyclin biosynthesis has recently been shown to be significantly enhanced in patients with severe atherosclerotic peripheral vascular disease who had evidence of platelet activation in vivo (180). In such patients, prostacyclin may therefore function as a locally-acting regulator of platelet-vascular homeostasis. Under these circumstances, preservation of prostacyclin synthesis is likely to be of particular importance and alternative pharmacological approaches require to be investigated.

### III. COMBINED INHIBITION OF PLATELET CYCLOOXYGENASE AND THROMBOXANE SYNTHASE

Combined inhibition of cyclooxygenase and thromboxane synthase represents a possible solution to the dilemma of developing a regimen which will maximally inhibit endogenous thromboxane generation without compromising prostacyclin formation. Such a combination is effective in vitro (363). In the study described above, aspirin in a dose of 120mg failed to inhibit thromboxane synthesis in vivo by more than 30% and, even in this dose, also caused a transient decline in prostacyclin biosynthesis. Addition of a thromboxane synthase inhibitor, which acts at a site distinct from aspirin in the synthetic cascade, should allow a greater degree of inhibition of thromboxane synthesis and will have no effect on vascular cyclooxygenase. Furthermore, if this hypothesis is correct, then further inhibition of platelet thromboxane synthesis in vivo, should be accompanied by accumulation of prostaglandin endoperoxides which can in turn be utilized by vascular tissue to generate prostacyclin. The effects of this approach on prostanoid formation and



platelet function in healthy subjects are discussed below. The thromboxane synthase inhibitor, UK 38,485, was given in a dose of 200mg which was shown to significantly increase prostacyclin synthesis in vivo when administered alone. The dose of aspirin coadministered with UK 38,485 was 120mg, which allowed direct comparison between the effects of the combined regimen and either drug alone.

#### Effects on Thromboxane Synthesis in vivo and ex vivo:

The effects on thromboxane synthesis in vivo of combined inhibition of cyclooxygenase and thromboxane synthase by single dose administration of aspirin (120mg) together with UK 38,485 (200mg) were assessed by measuring urinary Tx-M prior to dosing and in three post-dosing aliquots: 0-6, 6-12 and 12-24 hours. Tx-M excretion fell by a mean of  $58 \pm 7$  % from  $95 \pm 12$  to  $37 \pm 6$  pg/mg creatinine (Table 3.14). This reduction in Tx-M was significantly greater ( $p < 0.01$ ) than that seen after administration of either drug alone. No recovery of Tx-M excretion was seen during the 24-hour study period.

Serum thromboxane  $B_2$  was used as an index of

TABLE 3.14 Tx-M EXCRETION AFTER COMBINED CYCLOOXYGENASE AND THROMBOXANE SYNTHASE INHIBITION.

Time after dosing (hours)	Aspirin 120mg + UK 38,485 200mg	Aspirin 120mg	UK 38,485 200mg
-24	95±12	134±21	97±14
0-6	65±9*	108±18*	85±10
6-12	47±11* $\ddagger$	92±17*	56±6*
12-24	37±6* $\ddagger\ddagger$	104±17*	76±9

Values are expressed in pg/mg creatinine (mean  $\pm$  SEM) in 12 healthy male subjects.

\* p < 0.01 compared to control values.

$\ddagger$  p < 0.05 compared to aspirin 120mg or UK 38,485 200mg.

$\ddagger\ddagger$  p < 0.01 compared to aspirin 120mg or UK 38,485 200mg.

TABLE 3.15 SERUM THROMBOXANE B<sub>2</sub> AFTER COMBINED CYCLOOXYGENASE AND THROMBOXANE SYNTHASE INHIBITION.

Time after dosing (hours)	Aspirin 120mg + UK 38,485 200mg	Aspirin 120mg	UK 38,485 200mg
0	380±19	349±37	312±33
1	8±4 (98±1) $\ddagger$ **	18±4 (94±1)	12±3 (96±2)
4	8±4 (98±1) $\ddagger$ **	19±4 (94±1)	14±4 (96±1)
6	8±3 (98±1) $\ddagger$ **	24±7 (92±2)	31±6 (90±2)
8	14±4 (96±1)**	25±7 (29±3)	69±12 (78±4)
24	29±7 (92±2)**	26±7 (90±3)	129±23 (92±7)

Values are expressed as mean  $\pm$  SEM in ng/ml (% inhibition from pre-dose control) in 12 healthy male subjects.

$\ddagger$  p < 0.05 aspirin/UK 38,485 versus aspirin 120mg.

\*\* p < 0.01 aspirin/UK 38,485 versus UK 38,485 200mg.

thromboxane synthesis ex vivo and was measured in samples taken before and 1,4,6,8 and 24 hours after drug ingestion. The aspirin/UK 38,485 combination depressed serum thromboxane  $B_2$  generation by a mean of  $98 \pm 1\%$  from  $380 \pm 19$  to  $8 \pm 4$  ng/ml one hour after dosing (Table 3.15). Serum thromboxane  $B_2$  remained markedly inhibited throughout the 24-hour study period. The degree of inhibition was significantly greater than that achieved after either drug alone:  $p < 0.01$  1, 4, 6, 8 and 24 hours after dosing (aspirin/UK 38,485 versus UK 38,485 200mg) and  $p < 0.05$  1,4 and 6 hours after dosing (aspirin/UK 38,485 versus aspirin 120mg).

#### Effects on Prostacyclin Biosynthesis:

The combination of aspirin with UK 38,485 had no effect on prostacyclin biosynthesis as assessed by PGI-M excretion measured in the three aliquots (0-6,6-12 and 12-24 hours) after drug administration (Table 3.16). This contrasted with the significant increase in PGI-M caused by UK 38,485 administered alone and a similar decrease in PGI-M after aspirin.

TABLE 3.16 PGI-M EXCRETION AFTER COMBINED CYCLOOXYGENASE AND THROMBOXANE SYNTHASE INHIBITION.

TIME AFTER DOSING (hours)	Aspirin 120mg + UK 38,485 200mg	Aspirin 120mg	UK 38,485 200mg
-24	116±20	100±10	119±18
0-6	92±14	70±11**	122±16
6-12	111±23	104±22	143±19*
12-24	108±14	110±16	131±19

Values are expressed in pg/mg creatinine (mean ± SEM) in 12 healthy male subjects.

\*  $p < 0.05$ .

\*\*  $p < 0.01$

### Effects on Platelet Function:

The aspirin/UK 38,485 combination completely inhibited arachidonate-induced platelet aggregation and abolished the secondary wave of adrenaline-induced aggregation for the 24 hours of the study (Table 3.17). Platelet aggregation in response to high-dose collagen was significantly impaired to a similar degree to that seen for aspirin alone (Table 3.17). The bleeding time was also prolonged when measured one hour after dosing, from  $4.7 \pm 0.4$  to  $7.2 \pm 0.6$  minutes ( $p < 0.01$ ), but this was not significantly different from the increase seen after aspirin alone ( $p > 0.02$ ).

### Plasma Drug Levels:

Plasma drug levels of UK 38,485 were measured 1, 4, 6 and 8 hours after drug administration. Adequate plasma levels were achieved in all the subjects and no difference was apparent in the concentration of UK 38, 485 when it was co-administered with aspirin compared to those measured after UK 38,485 alone (Table 3.18).

**TABLE 3.17 EFFECTS ON PLATELET FUNCTION OF COMBINED CYCLOOXYGENASE AND THROMBOXANE SYNTHASE INHIBITION BY SINGLE DOSE ADMINISTRATION OF ASPIRIN 120mg PLUS UK 38,485 200mg.**

Time after dosing (hours)	PLATELET AGGREGATION IN PRP			Bleeding time (minutes)
	Arachidonic Acid (1.33mM)	Collagen (19µg/ml)	Adrenaline (10µM)	
0	0.76±0.06	2.00±0.08	15.9±0.4	4.7±0.4
1	NA	2.48±0.11*	PA	7.2±0.6*
4	NA	2.51±0.14*	PA	NT
6	NA	2.50±0.13*	PA	NT
8	NA	2.46±0.09*	PA	NT
24	NA	2.40±0.13*	PA	NT

Values are mean ± SEM of determinations in 12 healthy male subjects. Aggregation induced by arachidonic acid and collagen is measured as the lag time to achieving 50% of maximal aggregation (LT<sub>50</sub>, in minutes). Adrenaline-induced aggregation is measured as the height of the aggregation trace 6 minutes after addition of adrenaline (Tmax<sub>6</sub>, in cm).

\* p < 0.01.

NA = no aggregation.

PA = primary aggregation.

NT = not tested.

**TABLE 3.18 PLASMA CONCENTRATIONS OF UK 38,485 ADMINISTERED IN COMBINATION WITH ASPIRIN OR ALONE.**

Time after dosing (hours)	UK 38,485 (200mg) + Aspirin 120mg	UK 38,485 (200mg)
1	2992±320	3207±333
4	199±22	225±46
6	66±7	66±8
8	28±3	26±4

Values are expressed in ng/ml (mean ± SEM) in 12 healthy male subjects.

## Discussion

The combination of low-dose aspirin (120mg) with a thromboxane synthase inhibitor has been shown to inhibit thromboxane synthesis in vivo and ex vivo to a significantly greater extent than either drug administered alone. Although the difference in inhibition of serum thromboxane B<sub>2</sub> formation was small (94% for aspirin alone versus 98% for the combination), it was sufficient to enhance the extent of inhibition of actual thromboxane synthesis in vivo from 28% to almost 60%.

This observation provides additional evidence that the relationship between inhibition of serum thromboxane B<sub>2</sub> and Tx-M is not, in fact, linear. It also suggests that in patients with enhanced thromboxane biosynthesis, in whom treatment with such drugs might be expected to be of the most clinical benefit, inhibition of platelet capacity to generate thromboxane must be virtually complete before thromboxane synthesis in vivo is significantly reduced. Since maximal inhibition of platelet thromboxane production is apparently required to



prevent thromboxane-dependent platelet activation ex vivo (291) and in vitro (384), this has important clinical implications.

Despite the significantly greater decline in Tx-M after the aspirin/thromboxane synthase inhibitor combination, I was unable to demonstrate an enhancement of the anti-platelet effects of this regimen compared to those of aspirin. However, this is likely mainly to reflect platelet aggregation occurring by prostaglandin-independent mechanisms, since the response to high-dose collagen, though impaired, was largely maintained.

It is particularly interesting that the reduction in prostacyclin biosynthesis which was evident with low-dose aspirin, was no longer significant when the same dose of aspirin was administered in combination with UK 38,485. It is attractive to speculate that, as inhibition of endogenous thromboxane formation was incomplete after a single dose of 120mg of aspirin, the apparent lack of effect on prostacyclin biosynthesis may reflect the net effect of redirection of residual platelet endoperoxides to endothelial prostacyclin synthase. Thus, addition of a thromboxane synthase inhibitor appears to compensate for the inhibitory activity of aspirin against vascular

cyclooxygenase.

This study has therefore identified a potentially useful clinical approach to selective inhibition of thromboxane formation, although its effects during chronic administration remain to be determined. The discrepancy between the degree of inhibition of Tx-M and serum thromboxane  $B_2$  was again apparent. In view of the implications of this observation for platelet-inhibitory therapy in patients with thromboxane-dependent platelet activation in vivo, the relationship between inhibition of thromboxane synthesis in vivo and ex vivo is investigated further in the next section.

IV.FURTHER STUDIES ON THE RELATIONSHIP BETWEEN  
INHIBITION OF THROMBOXANE B<sub>2</sub> GENERATION  
IN SERUM AND Tx-M: Evidence that it is non-linear

It is well recognized that the capacity of tissues to generate icosanoids in response to physical and chemical stimuli far exceeds their actual biosynthetic rates in vivo (132)(181)(288)(390). Thus, although the capacity of platelets to generate thromboxane in clotting blood in response mainly to thrombin results in serum thromboxane B<sub>2</sub> levels of 300-400 ng/ml (336), the maximal endogenous concentration of thromboxane B<sub>2</sub> in plasma has been estimated at under 2 pg/ml (92). It follows, therefore, that the normal rates of thromboxane synthesis observed in vivo can be accounted for by activation of platelets to less than 0.1% of their capacity.

The studies described above suggest that there may also be a discrepancy between the degree of inhibition after aspirin or thromboxane synthase inhibitors of the platelet capacity for thromboxane generation in serum and of endogenous thromboxane biosynthesis. This observation may provide insight into the action of these drugs in human

syndromes of platelet activation. It also has important implications in the design of platelet-inhibitory therapy in such conditions since preservation of even a very small residual capacity to synthesize thromboxane may still, in response to stimuli in vivo, result in biologically-active levels of thromboxane.

In order to explore this further, I have examined the relationship between different degrees of inhibition of serum thromboxane  $B_2$  and the corresponding reduction in Tx-M excretion. In addition to using data obtained from the studies described above, I investigated the effect of a smaller dose of aspirin (20mg administered as 4 separate 5mg doses). This regimen was chosen to achieve an intermediate degree of inhibition of serum thromboxane  $B_2$ . The work of Patrono et al suggested that repeated dosing with 5 mg of aspirin should reduce serum thromboxane formation by around 50% (297).

#### Study Design:

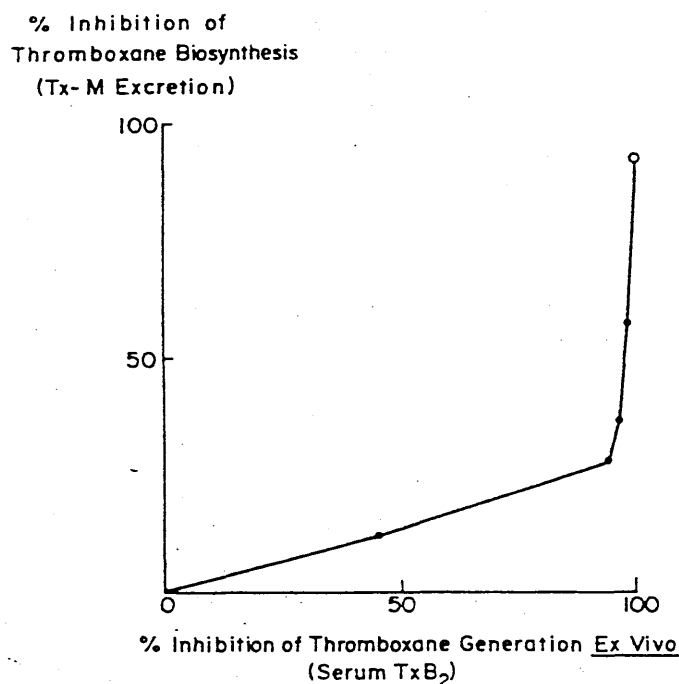
The effects on thromboxane synthesis in vivo and ex vivo of four different regimens which inhibit thromboxane formation were studied by measuring the maximal depression

of Tx-M excretion and serum thromboxane  $B_2$  in healthy volunteers. Regimens 1, 2 and 3, which are described in detail in sections I, II and III, were as follows: 1. UK 38,485 200mg, 2. aspirin 120mg and 3. aspirin 120mg plus UK 38,485, the drugs being administered as single doses in each case. Regimen 4 consisted of 20mg of aspirin administered orally as four 5mg doses taken two hours apart. Twelve healthy male subjects were studied in all parts of the study except in regimen 4 in which 4 subjects participated.

#### Results:

The small dose of aspirin (4x5mg) reduced serum thromboxane  $B_2$  by a maximum of  $48 \pm 2\%$  from  $365 \pm 21$  to  $190 \pm 8$  ng/ml 2 hours after the final dose. The corresponding fall in Tx-M was considerably smaller: from  $134 \pm 21$  to  $112 \pm 22$  pg/mg creatinine, representing a decline of  $14 \pm 4\%$ . These results are plotted together with those from regimens 1, 2 and 3 on Figure 3.7.

FIGURE 3.7 RELATIONSHIP BETWEEN INHIBITION OF SERUM THROMBOXANE  $B_2$  GENERATION EX VIVO AND Tx-M EXCRETION.



This shows that the relationship between peak inhibition of thromboxane generation ex vivo and peak inhibition of Tx-M departed markedly from the line of identity. Thus, as maximal blockade of the capacity to generate thromboxane ex vivo was approached, minor increments in the degree of inhibition achieved resulted in a disproportionately greater depression of thromboxane synthesis in vivo. This conclusion is supported by data derived from the only published study (carried out some years previously in the laboratory in which I worked) which is also included on the graph. In this study, complete inhibition of both serum thromboxane  $B_2$  (99%) and Tx-M excretion (97%) was obtained following chronic administration of 2,600mg of aspirin daily (291).

### Discussion:

Using several different regimens to achieve varying degrees of inhibition of thromboxane formation, I have shown that the relationship between the reduction in the capacity of platelets to generate thromboxane ex vivo and the reduction in thromboxane synthesis in vivo is non-linear. Indeed it departs markedly from the line of identity. The results show that minor increments (from 94% to 98%) in the extent of inhibition of serum thromboxane  $B_2$  give rise to much greater reductions in Tx-M excretion. The functional implications of this observation are illustrated by evidence that even a residual 10% capacity to generate thromboxane appears to be enough to fully sustain thromboxane-dependent platelet activation (384)(391). In addition, previous work has shown that it is possible to obtain further measurable inhibition of platelet function by increasing the inhibition of platelet thromboxane formation ex vivo from 95 to 99% (291).

Measurements of the platelet capacity to generate thromboxane are frequently used as a guide to dosing with platelet-inhibitory drugs (130)(290)(386)(392)(393)(394). The results of this study, however, indicate that this

will not accurately reflect the inhibition of actual thromboxane biosynthesis by platelets and consequently the effect on thromboxane-dependent platelet activation. It is clear that even when inhibition of serum thromboxane  $B_2$  is substantial (90-95%), thromboxane synthesis in vivo is largely maintained. Thus, in patients in which platelet activation in vivo plays an active role, it is likely that therapy should be directed towards achieving virtually complete inhibition of serum thromboxane  $B_2$  generation. Lesser degrees of inhibition would not be expected to substantially influence thromboxane-dependent platelet activation in such patients.



#### V. PRESYSTEMIC ACETYLATION OF PLATELET CYCLOOXYGENASE

An alternative approach to achieving selective inhibition of thromboxane formation was suggested by the observation that after oral administration of aspirin platelet thromboxane generation *ex vivo* appeared to fall significantly 10-15 minutes before aspirin was detectable in the plasma (395). Such an observation was consistent with acetylation by aspirin of platelet cyclooxygenase in the presystemic circulation. Aspirin is known to undergo substantial presystemic hydrolysis to salicylic acid in the intestine and liver before it enters the systemic circulation (396). During aspirin absorption from the gut platelets are therefore exposed, as they pass through the gut capillaries, to much higher concentrations of aspirin than in the systemic circulation. This raised the possibility that administration of an extremely low dose of aspirin might be sufficient to inhibit thromboxane formation in platelets as they passed through the presystemic circulation but, as hepatic extraction of such a small amount of aspirin should be virtually complete, pharmacologically active concentrations of aspirin would

not be achieved in the systemic circulation. Thus, the bulk of the vascular endothelium would not be exposed to aspirin and prostacyclin synthesis would be preserved.

Some support for the hypothesis that platelets were exposed to significant concentrations of aspirin in the presystemic circulation was provided by earlier studies investigating the pharmacology of enteric-coated "slow-release" aspirin preparations. These had shown inhibition of platelet cyclooxygenase and/or platelet aggregation in the absence of detectable plasma concentrations of aspirin in some subjects (382)(397)(398)(399)(400). The evidence remains presumptive, however, as the methods used to measure aspirin in these studies were relatively insensitive and the effects on vascular cyclooxygenase were not investigated.

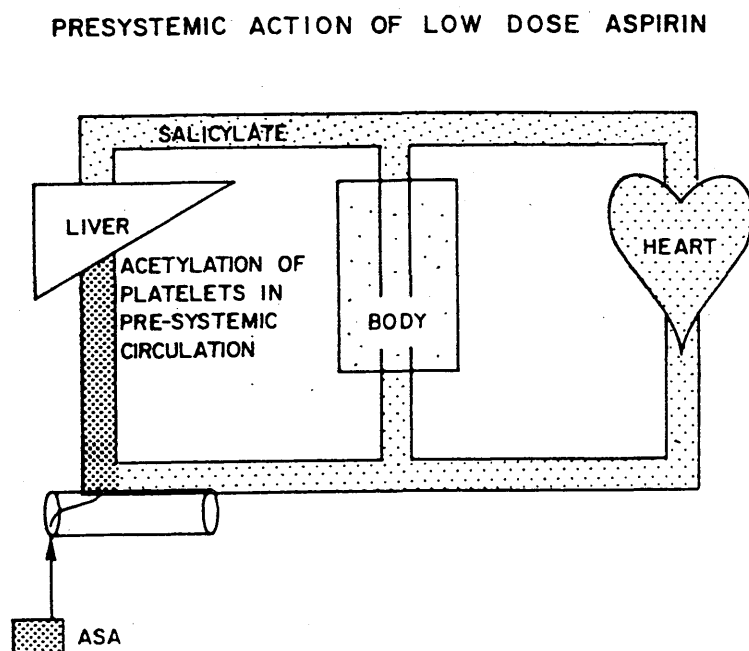
A model was therefore devised to test the hypothesis that repeated administration of extremely low doses of aspirin would result in cumulative inhibition of platelet cyclooxygenase in the presystemic circulation. Thus, even with chronic dosing regimens, the inhibitory effects of aspirin would be confined to platelets. The effects of such a regimen on vascular cyclooxygenase were assessed by measuring endogenous prostacyclin biosynthesis and a new

highly-sensitive GC/MS assay for aspirin and salicylate (360) was utilized to evaluate aspirin absorption and metabolism.

#### Design of the Model:

The model was designed to deliver extremely low doses of aspirin into the portal circulation over a prolonged period in healthy human subjects (Figure 3.8). The lowest possible dose (1mg) was chosen in order to minimize the chance of any aspirin reaching the systemic circulation.

FIGURE 3.8



Six male volunteers were studied. After an overnight fast single 1mg capsules of aspirin (prepared by Vanderbilt University Medical School pharmacy) were administered orally every 30 minutes to a total of 20 doses, thus mimicking a "slow-release" formulation. The aspirin was taken with a small amount of water and no meals were allowed during the study. The subjects had all refrained from ingesting aspirin or aspirin-like drugs for at least two weeks prior to the study.

Blood samples for measurement of serum thromboxane  $B_2$  and plasma aspirin and salicylate were taken immediately prior to and 5, 10, 15 and 30 minutes after the first dose of aspirin and then immediately prior to each subsequent dose. An additional sample was collected 30 minutes following the final dose which corresponded to 10 hours after the first dose of aspirin. In 4 of the subjects blood was also taken for platelet aggregation studies before and 2, 6 and 9 hours after the first dose of aspirin. Urine was collected for 24 hours before the study and in two post-dosing aliquots (0-12 hours and 12-24 hours) for measurement of the dinor metabolites of prostacyclin (PGI-M) and thromboxane (Tx-M). In addition, to assess the recovery of platelet thromboxane generation, serum thromboxane  $B_2$  was measured 1, 3, 7 and 10 days following the commencement of the study. For

comparative purposes the 6 subjects received, on a separate occasion, a single 20mg dose of aspirin administered orally after an overnight fast.

#### Effects on Platelet Thromboxane Formation:

Serum thromboxane  $B_2$  fell significantly within 5 minutes of the first 1mg dose of aspirin from  $267 \pm 54$  to  $221 \pm 45$  ng/ml ( $p < 0.05$ ). This represented a mean reduction of  $15 \pm 4$  % (Figure 3.9). The maximal reduction in serum thromboxane formation after 1mg of aspirin ( $22 \pm 9$  %) was seen 30 minutes following drug administration. There was a further, but much smaller, reduction in serum thromboxane  $B_2$  generation after the second 1mg dose to a mean of  $75 \pm 8$  % of pre-dosing values 60 minutes after the start of the study (Figure 3.9). With administration of subsequent 1mg doses, serum thromboxane  $B_2$  fell in a linear fashion by a maximum of  $55 \pm 4$  % to  $118 \pm 23$  ng/ml 30 minutes after the final 1mg dose of aspirin (Figure 3.10). In contrast to the "slow-release regimen" (SR-1), the single 20mg dose of aspirin (LD) depressed serum thromboxane by a maximum of  $33 \pm 7$  % (Figure 3.10). Tx-M excretion following the

FIGURE 3.9 SERUM THROMBOXANE B<sub>2</sub> AFTER ADMINISTRATION OF SIMULATED "SLOW-RELEASE" LOW DOSE ASPIRIN.

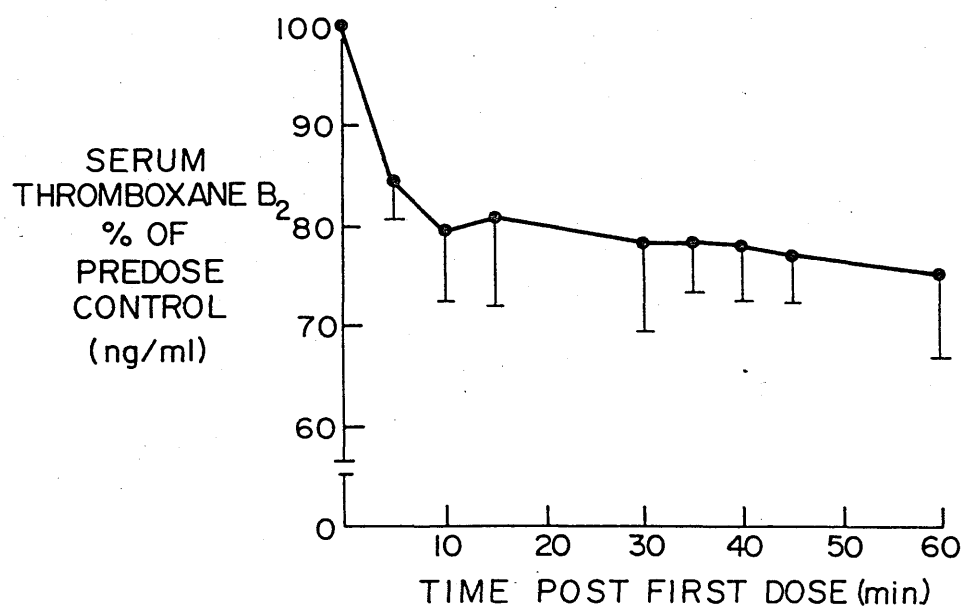
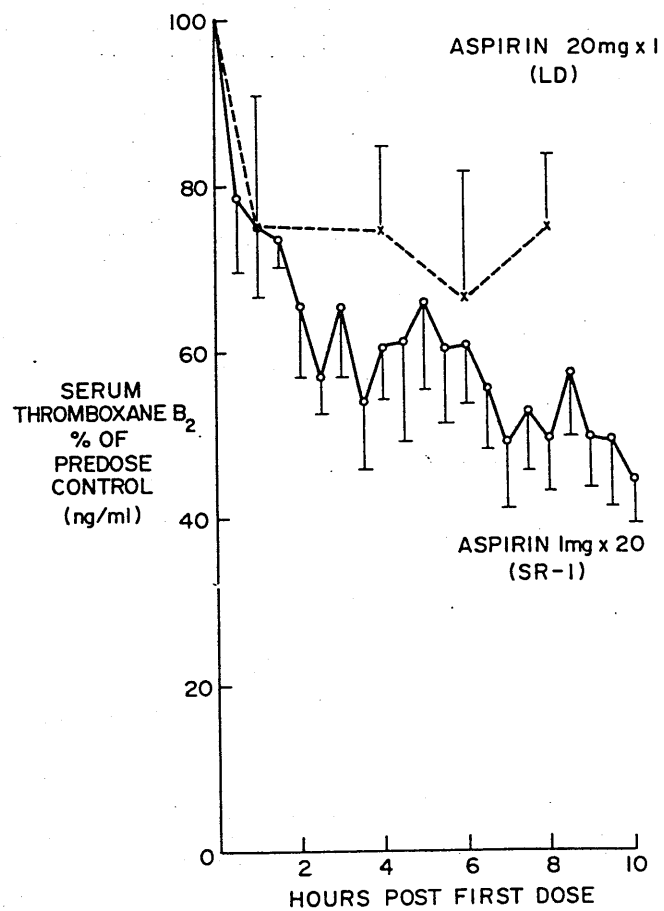


FIGURE 3.10 SERUM THROMBOXANE B<sub>2</sub> AFTER ADMINISTRATION OF A SINGLE DOSE OF ASPIRIN AND REPEATED ADMINISTRATION OF 20 1mg DOSES OF ASPIRIN.



"slow-release" aspirin regimen fell by a mean of  $19 \pm 11$  % from  $197 \pm 47$  to  $168 \pm 20$  pg/mg creatinine but this difference failed to reach statistical significance (Table 3.19).

TABLE 3.19 URINARY EXCRETION OF PROSTAGLANDIN METABOLITES (Tx-M AND PGI-M) AFTER ADMINISTRATION OF A "SLOW-RELEASE" LOW-DOSE ASPIRIN REGIMEN (SR-1).

	Tx-M	PGI-M
	(pg/mg creatinine)	
Before Aspirin:	$197 \pm 47$	$127 \pm 35$
After Aspirin		
0-12 hours:	$229 \pm 18$	$133 \pm 24$
12-24 hours:	$168 \pm 20$	$118 \pm 34$

Values are mean  $\pm$  SEM of determinations in 6 healthy male subjects.

#### Effect on Platelet Function:

Platelet aggregation in PRP was tested in response to 0.66mM arachidonic acid and 5uM adrenaline in 4 subjects after the SR-1 aspirin regimen. In one of the subjects (PI.) the secondary wave of adrenaline-induced aggregation disappeared and arachidonate-induced aggregation was



completely abolished after only 4mg of aspirin (Figure 3.11). In the remaining subjects there was some impairment of platelet aggregation after 4mg, but it was not until 9 hours following the first dose of aspirin (after a total of 18mg had been ingested), that significant inhibition of aggregation was seen in all subjects in response to both agonists (Table 3.20).

#### Evidence for a Presystemic Site of Action:

##### Plasma Aspirin and Salicylate Levels:

Aspirin was not detected in peripheral venous plasma at any time following administration of the SR-1 aspirin regimen, despite sampling every 5-15 minutes for the first hour and at 1/2-hourly intervals thereafter. The lower limit of sensitivity of the assay was 10 ng/ml. Absorption of aspirin was confirmed, however, by the demonstration of salicylate in venous plasma at the corresponding time points. After administration of the single 20mg dose (LD) of aspirin, the peak plasma aspirin concentration was 220 +/- 30 ng/ml measured 30 minutes after dosing.

FIGURE 3.11 ARACHIDONATE-INDUCED PLATELET AGGREGATION AFTER ADMINISTRATION OF 4 1mg DOSES OF ASPIRIN.

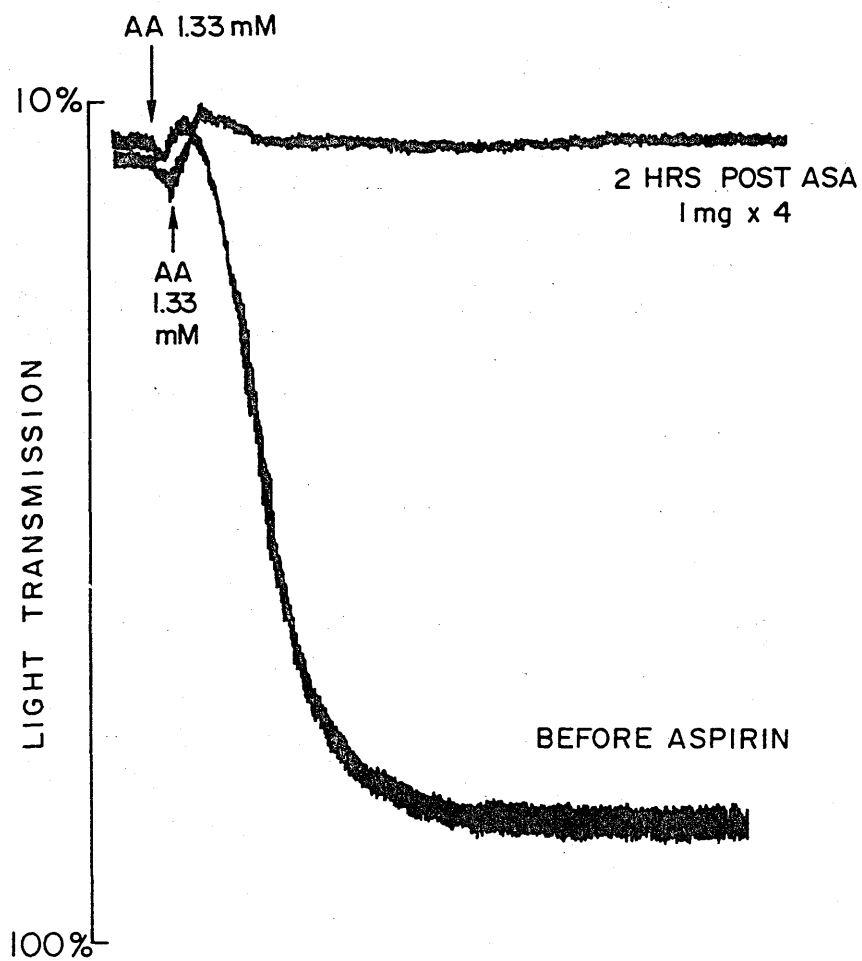


TABLE 3.20 PLATELET AGGREGATION IN PRP AFTER ADMINISTRATION OF A "SLOW-RELEASE" LOW-DOSE ASPIRIN REGIMEN (SR-1).

Subjects	Arachidonic Acid 0.66mM (LT <sub>50</sub> , minutes)				Adrenaline 5µM (Tmax <sub>6</sub> , cm)			
	Time after dosing (hours)				Time after dosing (hours)			
	0	2	6	9	0	2	6	9
PL	1.13	NA	NA	NA	18.4	PA	PA	PA
MW	1.04	0.96	2.40	NA	18.4	PA	PA	PA
SB	1.68	1.84	NA	NA	15.2	18.2	16.9	PA
AC	1.12	1.20	0.80	2.30	9.2	15.6	14.9	PA

NA : no aggregation

PA : primary wave of aggregation

LT<sub>50</sub> : lag time to achieving 50% of maximal aggregation

Tmax<sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist

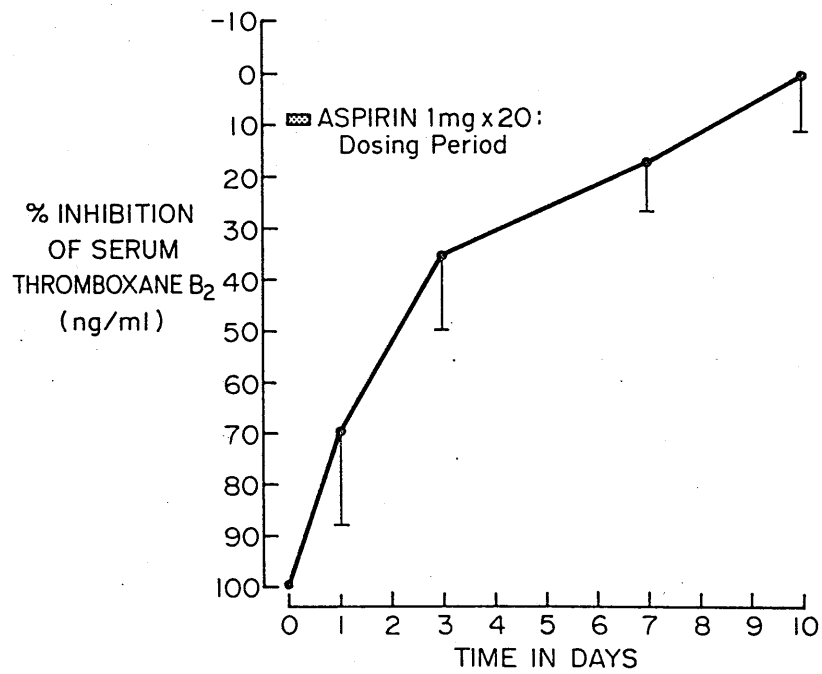
### Effect on Prostacyclin Biosynthesis:

PGI-M excretion was  $127 \pm 35$  pg/mg creatinine prior to dosing with the SR-1 aspirin regimen and was not significantly different in the 2 post-dosing aliquots (Table 3.19).

### Pattern of Recovery of Serum Thromboxane $B_2$ :

The pattern of recovery of serum thromboxane  $B_2$  formation after the SR-1 aspirin regimen is shown in Figure 3.12. The lowest value of serum thromboxane, which was measured 30 minutes after the final 1mg dose, represents 100% inhibition (ie 0% recovery) and full recovery to predosing values is thus equivalent to 0% inhibition. Recovery of the platelet capacity for thromboxane synthesis began within 24 hours: at 14 hours after the last dose serum thromboxane  $B_2$  had recovered by just over 30% to  $204 \pm 66$  ng/ml and reached normal values 9 days later.

FIGURE 3.12 RECOVERY OF SERUM THROMBOXANE B<sub>2</sub> AFTER ADMINISTRATION OF SIMULATED "SLOW-RELEASE" LOW-DOSE ASPIRIN.



## Discussion

The purpose of this study was to establish a human model of selective inhibition of thromboxane synthesis using extremely low doses of aspirin to inactivate platelet cyclooxygenase in the presystemic circulation. Since aspirin undergoes substantial presystemic hydrolysis to salicylate in the intestine and liver (396), very small doses of aspirin are likely to be almost completely metabolized. Thus, negligible amounts of intact aspirin should reach the systemic circulation. In this way vascular cyclooxygenase would remain unexposed to inhibitory concentrations of aspirin and prostacyclin biosynthesis would consequently be preserved. A simulated "slow-release" mode of administration was used to deliver extremely small quantities of aspirin into the portal circulation in a controlled manner. While a single low-dose enteric-coated aspirin formulation would have been much more convenient, such a preparation was not available in a low enough dose. Perhaps more importantly, erratic absorption of enteric-coated aspirin preparations (359)(382)(397)(399)(401)(402) with the possibility of intermittently larger increments in aspirin bioavailability was likely to make interpretation of the

results very difficult.

In order to ensure that hepatic extraction of aspirin would be virtually complete and minimize the chance of significant amounts of aspirin reaching the systemic circulation, the smallest practicable dose was employed in this study. The results show that even in doses as low as 1mg aspirin is able to induce measurable inhibition of the capacity of platelets to generate thromboxane. Since it irreversibly acetylates the cyclooxygenase enzyme and platelets are unable to synthesize new enzyme, the inhibitory effects of aspirin on platelet thromboxane generation are cumulative on repeated dosing (296)(297)(333). Thus, although serum thromboxane  $B_2$  fell by a mean of 22% after a single 1mg dose of aspirin, there was a progressive decline in thromboxane generation with repeated administration of 1mg until it had fallen by 55% after a total of 20mg. This contrasted with a maximal reduction in serum thromboxane  $B_2$  after a single 20mg dose of 33%.

That the effects of the "slow-release" aspirin regimen were the result of presystemic acetylation of platelet cyclooxygenase was suggested by three lines of evidence. Firstly, significant cumulative inhibition of serum thromboxane  $B_2$  generation and thromboxane-dependent

platelet aggregation was achieved in the absence of detectable plasma concentrations of aspirin. Secondly, prostacyclin biosynthesis was unaltered. Thirdly, the recovery of the platelet capacity to generate thromboxane was accelerated.

Several previous studies have shown that despite undetectable plasma levels of aspirin, enteric-coated slow-release preparations of aspirin may inhibit platelet cyclooxygenase activity (382)(397)(399). Others have been able to demonstrate significant inhibition of platelet aggregation in the absence of measurable concentrations of aspirin in peripheral plasma (382)(398)(400). However, the doses used in these studies have generally been high (650-1300mg) and in every study the methods used to quantitate plasma concentrations of aspirin have been relatively insensitive, with detection levels 10-100 fold less than the assay employed in the present investigation. Indeed, similar doses of slow-release aspirin have been reported by several other workers to give rise to significant plasma aspirin concentrations (402)(403)(404)(405)(406)(407). It is difficult, therefore, to interpret the significance of "undetectable" levels of aspirin in these earlier studies, particularly when in some cases the reported assays were unable to measure aspirin in peripheral blood even after a conventional



320mg tablet of compressed aspirin (399). In the present study plasma salicylate levels confirmed absorption of each lmg dose of aspirin and as an additional control aspirin was readily detectable in peripheral plasma after single dose administration of 20mg of aspirin.

Further evidence in support of a presystemic site of action for the "slow-release" aspirin regimen employed in the present study is provided by the lack of effect of this regimen on endogenous prostacyclin biosynthesis. This is not conclusive evidence, however, as single dose administration of 20mg of aspirin has also been shown to have no significant effect on prostacyclin formation in vivo (65). Since chronic administration of such a dose does inhibit prostacyclin synthesis in vivo (291) and ex vivo (386), confirmation of the ability of very low doses given in a "slow-release" form to preserve prostacyclin biosynthesis requires to be tested in chronic dosing studies. Nevertheless, since there was no evidence of accumulation of aspirin after 20 lmg doses given at half-hourly intervals, it is very unlikely that prostacyclin synthesis would be depressed even with long-term administration. Although significant accumulation of salicylate is likely to occur with chronic dosing, this has been shown to have no inhibitory effects on endogenous prostacyclin production even in very high

doses (53 mg/kg) (383).

The accelerated rate of recovery of serum thromboxane  $B_2$  generation observed in all the subjects in the present study was also consistent with the model of presystemic acetylation of platelet cyclooxygenase by the "slow-release" aspirin regimen. It is generally believed that the normal 48-hour lag in the recovery of platelet capacity for thromboxane synthesis (286)(296)(408)(409) is due to acetylation of megakaryocyte cyclooxygenase by aspirin (410). The substantial recovery of serum thromboxane  $B_2$  occurring within 15 hours of the last dose of aspirin is therefore highly suggestive of a failure to acetylate megakaryocytes which would not be exposed to inhibitory concentrations of aspirin.

Despite an overall reduction of thromboxane generation in serum of more than 50% after the simulated "slow-release" aspirin regimen, the decline in Tx-M excretion was much smaller, less than 20%, and failed to reach statistical significance. This is consistent with the studies described above in which the relationship between these two indices was shown to be non-linear. In addition it may reflect the partial recovery of platelet thromboxane generation which began within the initial 24 hour study period. The reduction in platelet thromboxane

generation was, however, sufficient to produce measurable inhibition of platelet function, in most individuals after only 4-12 mg of aspirin. Others have recently reported that chronic administration of 1mg aspirin daily to healthy volunteers for several weeks is sufficient to render platelets more sensitive to the anti-aggregatory effects of prostacyclin in vitro (411) and to induce small (15%) but significant reductions in serum thromboxane  $B_2$  (412).

This study has used repeated oral administration of an extremely low dose of aspirin to establish a model of selective inhibition of thromboxane formation by acetylation of platelet cyclooxygenase in the presystemic circulation. Such a regimen was not only selective with respect to its effect on prostacyclin production, but also achieved a greater degree of inhibition of platelet thromboxane generation than did an equivalent amount of aspirin administered as a single dose. The potential clinical benefit of such an approach would depend on the feasibility of developing a sustained-release preparation which was able to deliver an appropriate low dose of aspirin at a controlled rate, although a similar objective might be possible using continuous infusion of aspirin in minute amounts. These regimens could therefore be used to more rapidly attain complete inhibition of platelet

thromboxane synthesis which, as discussed above, is likely to be necessary to prevent thromboxane-dependent platelet activation in a clinical setting. They would have the additional benefit of protecting not only vascular cyclooxygenase from the potentially harmful effects of aspirin, but also other sources of the enzyme, particularly renal and gastric cyclooxygenase, which may otherwise limit the therapeutic use of aspirin (413).

## CONCLUSION

The biochemical and functional effects of several different approaches to achieving selective inhibition of thromboxane formation have been investigated in healthy subjects. An important objective was to identify a regimen which satisfied the dual criteria of maximising the reduction in thromboxane synthesis without a corresponding fall in prostacyclin production. The various regimens studied demonstrated varying degrees of selectivity.

The thromboxane synthase inhibitor efficiently blocked platelet thromboxane synthesis ex vivo and, in the higher dose, also significantly increased prostacyclin biosynthesis. Nevertheless, the effects on platelet function were relatively minor and actual thromboxane synthesis in vivo fell by less than 40%. The significantly greater platelet-inhibitory activity of the low-dose aspirin regimen (120mg) and in vitro studies of platelet behaviour with thromboxane synthase inhibitors suggest that accumulated prostaglandin endoperoxides substitute

for the proaggregatory effects of thromboxane when arachidonate metabolism via thromboxane synthase is blocked. Even in this dose, which is within the range conventionally accepted as "low-dose aspirin", aspirin caused a transient but significant reduction in endogenous prostacyclin formation and the corresponding decline in thromboxane biosynthesis was far from optimal (around 30%). These results indicate that, despite contentions to the contrary, a truly selective single dose of aspirin is not achievable.

However, two new and potentially useful approaches were identified which were both highly selective and caused measurable inhibition of platelet function in all the subjects studied. The combination of low-dose aspirin with a thromboxane synthase inhibitor appeared to offset the principal disadvantages of either drug administered alone. Thus, the fall in prostacyclin seen after low-dose aspirin was no longer apparent with the combined regimen and there was substantial and prolonged inhibition of platelet function. Furthermore, the inhibitory effect on endogenous thromboxane formation appeared to be additive (a reduction of almost 60%) in response to enzyme blockade at two different steps in its biosynthetic pathway. The other novel regimen employed repeated administration of extremely low doses of aspirin to achieve selective

presystemic acetylation of platelet cyclooxygenase. These latter two approaches remain to be fully evaluated, however, to determine the optimum formulations and dosing schedules for chronic drug administration.

The studies described in this chapter were the first to investigate the effects of these drugs on actual thromboxane synthesis in vivo using a modified, non-invasive, highly sensitive and specific assay for a major urinary metabolite of thromboxane, 2,3-dinor-thromboxane  $B_2$  (Tx-M). As a result, they highlighted a previously unrecognized but important observation with significant implications for platelet inhibitory therapy. It is apparent that the relationship between inhibition of the platelet capacity for thromboxane generation, as reflected by serum thromboxane formation ex vivo, and actual thromboxane synthesis in vivo is not linear. Thus, since the capacity of platelets to produce thromboxane in response to diverse physical and chemical stimuli far exceeds the actual rates of thromboxane synthesis in vivo, inhibition of this capacity is likely to have to be virtually complete to prevent thromboxane-dependent platelet activation. It is also clear that the therapeutic potential of strategies designed to inhibit endogenous thromboxane formation will therefore have to be investigated in human models of platelet

activation in vivo in which thromboxane-dependent platelet activation plays a significant role. The studies in the following chapters focus on the identification of appropriate models and evaluate the effects of thromboxane synthesis inhibition in one of them.

I acknowledge the help of Ms Johniene Doran with some of the platelet function tests carried out in these studies.



## Chapter 4

### THROMBOXANE BIOSYNTHESIS IN HUMAN SYNDROMES OF PLATELET ACTIVATION

## INTRODUCTION

### I. SEVERE ATHEROSCLEROSIS

Study Design:

Patient selection

Analytical methods

Evidence for Platelet Activation in vivo

Thromboxane Biosynthesis (Tx-M)

Discussion

### II. SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON

Study Design:

Patient selection

Analytical methods

Evidence for Platelet Activation in vivo

Effect of Cooling

Discussion

### III.MYELOPROLIFERATIVE DISORDERS

Study Design:

Patient selection

Analytical methods

Results

Discussion

## INTRODUCTION

Thromboxane  $A_2$  has been implicated as an important mediator in diverse human diseases in which platelet activation and/or vasospasm are known to occur (see Table 1.1). While this is an attractive hypothesis, documented evidence of enhanced thromboxane biosynthesis in such conditions has been lacking. As discussed in chapter one, this is largely due to methodological problems which are inherent to the measurement of such evanescent compounds, particularly when invasive sampling procedures are employed (130). However, measurement of a major urinary metabolite of thromboxane, Tx-M, circumvents most of these difficulties and has recently allowed the rate of secretion of thromboxane into the circulation to be calculated (92). Under physiological conditions in humans it appears to be very low- in the order of 1-2 pg/ml (92). This is not surprising since eicosanoids are not stored within cells but are synthesized following a stimulus which releases arachidonic acid from the cell membrane. Thus, in healthy individuals in the absence of stimuli which trigger platelet activation endogenous production of thromboxane would be expected to be low.

Conversely, thromboxane biosynthesis would be predicted to be increased in the presence of platelet activation in vivo.

The following series of studies also employs quantitation of Tx-M in urine to investigate endogenous thromboxane biosynthesis in various syndromes in which platelet activation in vivo is believed to occur. I elected to study in detail three groups of conditions which, on the basis of preliminary experiments outlined in chapter 2, were associated with elevations of Tx-M excretion and platelet activation in vivo: severe atherosclerosis, systemic sclerosis with Raynaud's phenomenon and myeloproliferative disorders. The presence of platelet activation in these patients was determined using a number of markers of platelet function, including measurement of platelet granule proteins, circulating platelet aggregates and platelet aggregation. Although the interpretation of all of these methods may be confounded by the influence of ex vivo activation (263)(265)(355), this was controlled for by obtaining matching data from healthy subjects for each group of patients.

Several animal models of platelet activation have been established and have provided a useful approach to the evaluation of platelet-inhibitory therapy

(203)(343)(344)(346). In man no equivalent model has been defined. The aim of these studies was not only to enhance our understanding of the pathogenesis of such conditions, but also to identify appropriate clinical targets for therapeutic strategies designed to inhibit thromboxane-dependent platelet activation.

## I. SEVERE ATHEROSCLEROSIS

The role of platelets in the pathogenesis of atherosclerosis remains controversial. Nevertheless, evidence from pathological, biochemical and clinical studies supports the contention that platelet activation is an important factor in acute thrombotic events and in the evolution of diffuse atherosclerotic disease (190)(193)(194)(414). Direct evidence from post-mortem studies shows that platelets constitute the major component of occlusive thrombi in atherosclerotic vessels (191)(415) and that in acutely thrombosed arteries the thrombus which has precipitated luminal occlusion consists largely of aggregated platelets (192). In addition to their involvement in occlusive thrombi, platelets may contribute to the formation of the proliferative lesions of atherosclerosis by producing growth factors which stimulate smooth muscle cell proliferation (416)(417). The measurement of diverse biochemical indices of platelet activation in vivo provides supportive evidence for the involvement of platelets and their products in patients with longstanding atherosclerotic disease (233)(350)(354)(418)(419)(420).

The following study was designed to investigate thromboxane biosynthesis in a well-defined group of patients with severe atherosclerosis and evidence of platelet activation in vivo. The patients all had advanced diffuse obliterative arterial disease of the lower limb and were selected on the basis of preset criteria indicative of platelet activation. This group of patients was considered unsuitable for reconstructive vascular surgery and, since it had not at the time been shown to be of proven value, was not on treatment. They therefore represented an appropriate stable model of severe atherosclerosis in which to test the hypothesis that platelet activation in vivo is associated with enhanced endogenous formation of thromboxane.

### Study Design

#### Patient selection:

The nine patients studied (8 men and one woman, aged 49-75 years) were selected from the peripheral vascular disease clinics of Vanderbilt University Medical School



and the Veterans Administration Hospital, Nashville, Tennessee. All had angiographically confirmed obstructive arterial disease of the lower limbs with pain at rest and/or ischaemic ulcers (grades III and IV). They met preset criteria for the presence of platelet activation in vivo: plasma B-thromboglobulin > 20 ng/ml and a circulating platelet aggregate (CPA) ratio of more than or equal to 1.3. Since previous work has shown that spurious increases in B-thromboglobulin can be identified by the presence of concomitantly high levels of platelet factor 4 (265), samples with platelet factor 4 levels above 18 ng/ml were excluded. These values were predetermined on the basis of the range of values obtained in over 30 healthy individuals over a wide age range (21-88 years) (see Appendix II). Five of the patients had insulin-dependent diabetes and seven had electrocardiographic evidence of myocardial ischaemia, although only one was taking anti-anginal medication (occasional sublingual glyceryl trinitrate). Seven of the nine patients studied were chronic cigarette smokers. None had taken aspirin or other nonsteroidal anti-inflammatory drugs for at least 10 days before the study.

As shown in Appendix 1, excretion of the urinary metabolites of thromboxane and prostacyclin increases significantly with advancing age. Therefore to serve as

age-matched controls, ten apparently healthy volunteers (age 50-75 years) working in the above hospitals or attending a local Geriatric Centre were studied in parallel. None was on regular therapy and all denied a significant past history of disease, in particular cerebrovascular, cardiovascular, peripheral vascular or renal disease.

#### Analytical methods:

The methods used were as described in chapter 2. Venous blood was collected without stasis via a 19-gauge butterfly needle. Plasma was obtained for measurement of B-thromboglobulin and platelet factor 4 by radioimmunoassay and for the determination of the circulating platelet aggregate ratio by the modified method of Wu and Hoak (350). Serum thromboxane  $B_2$  was measured by radioimmunoassay to confirm that no aspirin had recently been taken. In addition, the bleeding time was measured using the template method. Urine was collected in 24-hour aliquots for quantitation of the major urinary metabolites of prostacyclin (2,3-dinor-6-keto-PGF<sub>1 $\alpha$</sub> , PGI-M) and thromboxane (2,3-dinor-thromboxane  $B_2$ , Tx-M) by gas chromatography/mass spectrometry using the stable isotope

dilution assays described in chapter 2.

#### Evidence for Platelet Activation in vivo:

All the patients fulfilled the preset criteria consistent with the presence of platelet activation in vivo. Plasma B-thromboglobulin was markedly elevated at  $113 \pm 22$  ng/ml compared to  $13 \pm 3$  ng/ml in the healthy control subjects ( $p < 0.001$ ) (Figure 4.1). In addition, the CPA ratio was significantly higher in the patients compared to the controls ( $1.4 \pm 0.01$  vs  $1.0 \pm 0.02$ ;  $p < 0.05$ ). The levels of platelet factor 4 measured in the same samples were within the normal range:  $11.7 \pm 0.5$  versus  $11.0 \pm 2$  ng/ml (patients vs controls). The atherosclerotic patients also had a significantly shorter bleeding time ( $2.9 \pm 0.2$  vs  $5.0 \pm 0.4$  minutes;  $p < 0.001$ ) (Figure 4.1).

Figure 4.2 shows the urinary excretion of PGI-M for the atherosclerotic patients and the healthy age-matched controls. Normal values for healthy young subjects are included for comparison. Prostacyclin biosynthesis was significantly enhanced in the patients compared to the

FIGURE 4.1 INDICES OF PLATELET ACTIVATION IN VIVO IN PATIENTS WITH SEVERE ATHEROSCLEROSIS.

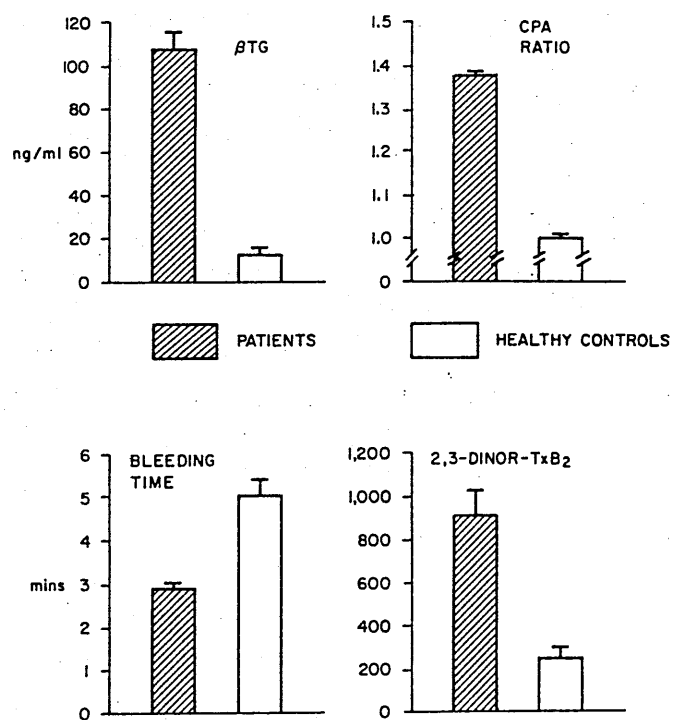


FIGURE 4.2 PGI-M EXCRETION IN PATIENTS WITH SEVERE ATHEROSCLEROSIS AND HEALTHY CONTROL SUBJECTS.

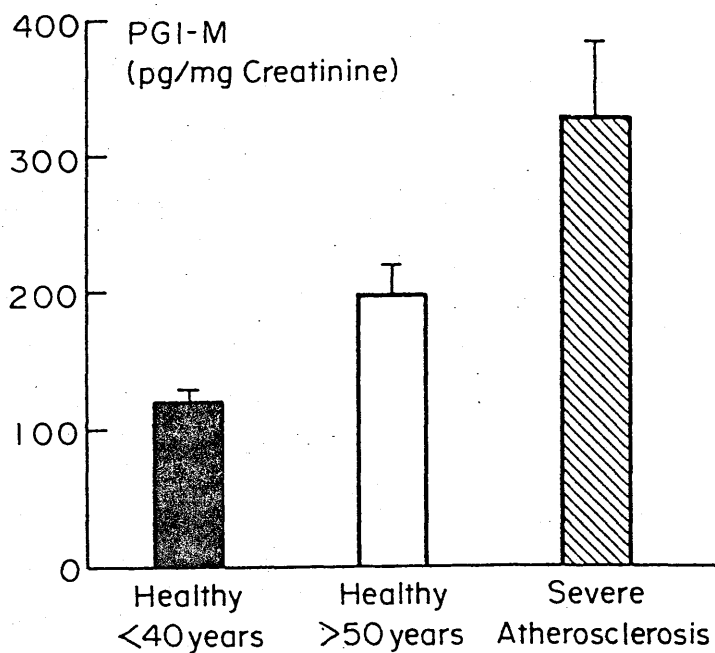
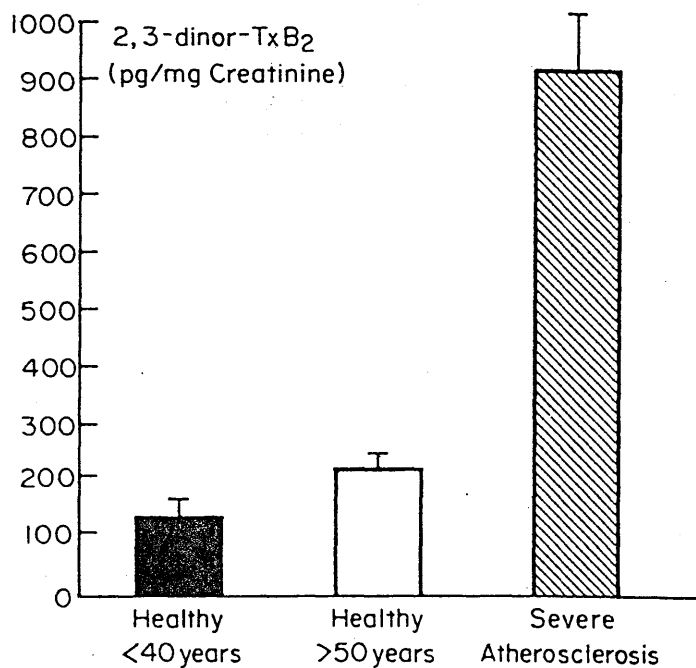


FIGURE 4.3 Tx-M EXCRETION IN PATIENTS WITH SEVERE ATHEROSCLEROSIS AND HEALTHY CONTROL SUBJECTS.



age-matched controls (323 +/- 89 vs 197 +/- 21 pg/mg creatinine;  $p < 0.05$ ), providing further indirect evidence of platelet activation in vivo in this model. Serum and urine creatinine were within normal limits in all the subjects studied.

#### Thromboxane Biosynthesis (Tx-M):

Urinary excretion of Tx-M was markedly increased in the patients with severe atherosclerosis compared to healthy controls (Figure 4.1). Normal values for healthy young controls are shown for comparison (Figure 4.3). Tx-M in the patients ranged from 484 to 1816 pg/mg creatinine with a mean of 912 +/- 99 pg/mg creatinine. This was significantly greater ( $p < 0.001$ ) than mean Tx-M excretion in the healthy age-matched controls (223 +/- 22 pg/mg creatinine). However, there was no significant difference between patients and age-matched controls in thromboxane  $B_2$  generation in serum (300 +/- 46 vs 340 +/- 45 ng/ml; patients vs controls) or in the peripheral blood platelet counts (292 +/- 19 vs 298 +/- 32  $\times 10^9/l$ ).

## Discussion

This study has demonstrated markedly enhanced endogenous formation of thromboxane in patients with advanced atherosclerosis in whom there was also evidence of platelet activation in vivo. Although, as I showed in the earlier studies, Tx-M is mainly derived from platelets in healthy individuals, the source of the increase in Tx-M was not directly addressed in this study. However, the following lines of evidence suggest that it is most likely to be of platelet origin. Firstly, the increase in Tx-M was accompanied by other markers of platelet activation in vivo, including a substantially elevated plasma concentration of B-thromboglobulin and the presence of circulating platelet aggregates. Secondly, prostacyclin biosynthesis was also enhanced in these patients. Increased prostacyclin production has previously been demonstrated in patients with severe atherosclerosis and platelet activation and is thought to reflect enhanced platelet-vascular interactions in this setting (180). In addition, the concomitant increase in PGI-M suggests that the observed enhancement of Tx-M excretion is not simply the result of enhanced metabolism to the dinor metabolite of a normal amount of thromboxane (the fractional

conversion of prostacyclin to its dinor metabolite is normal in this setting (180)). Since renal function was normal in both patients and controls, it also seems unlikely that altered renal handling of the metabolite accounted for the marked discrepancy between the levels of Tx-M in the two groups of subjects.

In order to clarify the relationship between enhanced synthesis of Tx-M and platelet activation in vivo, it is necessary to examine the effects on Tx-M both of inducing and of inhibiting platelet activation. Previous work in animal models of acute thrombosis has shown increases in plasma thromboxane  $B_2$  in response to various stimuli, including arachidonate-induced myocardial ischaemia (202) or sudden death (211) and experimentally stenosed coronary artery occlusion (206). More recently, Fitzgerald et al have developed a canine model of platelet-dependent coronary occlusion in which complete obstruction of the coronary artery is accompanied by a marked increase in urinary Tx-M (421). In addition, coronary occlusion is prevented or significantly delayed by administration of specific endoperoxide/thromboxane  $A_2$  receptor antagonists. Since then the same workers have also reported phasic increases in Tx-M excretion in patients with unstable angina during episodes of acute chest pain (422), a situation in which platelet thrombi



have been demonstrated in the coronary vessels (423).

Thus, Tx-M appears to be a sensitive, non-invasive index of platelet activation in vivo which may be elevated in chronic disease, such as severe atherosclerosis, and increase further in response to an acute stimulus. The effects of inhibiting endogenous thromboxane formation in such patients, either directly using a specific inhibitor of thromboxane synthesis or indirectly by using an inhibitor of platelet activation which has no specific activity on the thromboxane biosynthetic pathway remain to be investigated. One preliminary report, in which platelet activation was inhibited indirectly using prostacyclin, did show a decline in Tx-M excretion from initially elevated levels in a patient with thrombotic thrombocytopenic purpura (341). The present study has identified a clinically relevant, stable model of in vivo platelet activation in which to investigate the effects of platelet-inhibitory therapy on thromboxane biosynthesis.

## II.SYSTEMIC SCLEROSIS WITH RAYNAUD'S PHENOMENON

Raynaud's phenomenon is characterised by episodic exaggerated constriction of the small digital arterioles and arteries in response to cold or emotional stress (424). In patients with Raynaud's phenomenon associated with systemic sclerosis marked structural changes have been demonstrated in the blood vessels, particularly intimal hyperplasia and severe fibrosis (425)(426). In addition, other investigators have found evidence of platelet activation in vivo in patients with this disease, including the presence of circulating platelet aggregates and elevated plasma B-thromboglobulin levels (427)(428)(429). It was therefore attractive to speculate that such patients might provide a further human model of platelet activation in vivo in which thromboxane biosynthesis was likely to be enhanced.

The aims of this study were firstly to measure Tx-M excretion in a group of patients with longstanding systemic sclerosis complicated by Raynaud's phenomenon who had evidence of in vivo platelet activation; and secondly, to determine whether induction of an acute attack in

response to exposure to cold was accompanied by a further increase in Tx-M formation.

#### Study Design:

#### Patient selection:

The ten patients studied (9 women and one man, aged 36-60 years) all had systemic sclerosis complicated by symptomatic Raynaud's phenomenon and were attending the outpatient clinic at Vanderbilt University Medical Center. Each patient fulfilled the criteria of the American Rheumatism Association for a diagnosis of systemic sclerosis (430), all were non-smokers and none had taken non-steroidal anti-inflammatory drugs within the two weeks prior to the study. Any patients with a clinical history or laboratory evidence of ischaemic heart disease (ECG) or renal impairment (serum creatinine > 133  $\mu\text{mol/l}$ ) were excluded from the study.

Ten healthy age- and sex-matched volunteers served as controls. The patients and the control subjects were studied before and after central cooling had been induced with a cooling blanket (Aquamatic-K-thermia, Gorman-Rupp,

Belville, Ohio). Cooling was continued for 30 minutes or until Raynaud's phenomenon developed, which was observed in all the patients. Care was taken to ensure that the maximum fall in the subject's normal oral temperature did not exceed 3°F.

#### Analytical methods:

Blood was taken for measurement of plasma B-thromboglobulin and platelet factor 4, the circulating platelet aggregate (CPA) ratio, serum thromboxane  $B_2$  and platelet aggregation in response to arachidonic acid. B-thromboglobulin, platelet factor 4 and serum thromboxane  $B_2$  were measured by radioimmunoassay as described in chapter 2 and the CPA ratio was determined using a modification of the method of Wu and Hoak (350). Platelet aggregation was studied in PRP using the light transmission method. The threshold aggregating concentration for arachidonic acid was determined (defined as the minimum concentration producing a greater than 70% increase in light transmission). Endogenous biosynthesis of thromboxane and prostacyclin was assessed by measuring their major metabolites in urine, Tx-M (2,3-dinor-thromboxane  $B_2$ ) and PGI-M (2,3-dinor-6-keto-PGF<sub>1 $\alpha$</sub> ), by gas chromatography/mass

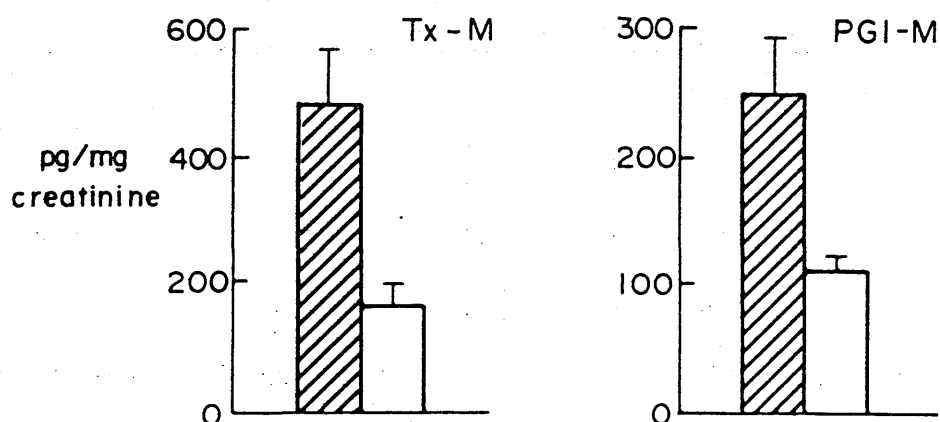
spectrometry. To determine the effect of cooling, urine was collected from both patients and controls for 24 hours prior to cooling and in an aliquot corresponding to the time of cooling for measurement of Tx-M and PGI-M. Blood was also taken at the end of the period of cooling for plasma B-thromboglobulin, platelet factor 4 and platelet aggregation studies.

#### Evidence for Platelet Activation in vivo:

Basal excretion of Tx-M was significantly increased ( $p < 0.001$ ) in the patients with systemic sclerosis and Raynaud's phenomenon ( $486 \pm 88$  pg/mg creatinine) compared to the healthy control subjects ( $162 \pm 38$  pg/mg creatinine) (Figure 4.4). PGI-M excretion was also significantly higher ( $p < 0.005$ ) in the patients compared with the controls ( $248 \pm 39$  vs  $112 \pm 10$  pg/mg creatinine). There was a marked increase in plasma B-thromboglobulin in the patients with systemic sclerosis ( $57 \pm 16$  vs  $14 \pm 2$  ng/ml;  $p < 0.005$ ). However, plasma platelet factor 4 levels were also significantly higher in the patients ( $43 \pm 14$  vs  $6 \pm 1$  ng/ml;  $p < 0.005$ ).

suggesting that there was considerable platelet activation occurring ex vivo. Since normal platelet factor 4 levels were obtained in the control samples processed in an identical manner, and since the CPA ratio was also increased in the patients ( $1.43 \pm 0.11$  vs  $1.04 \pm 0.03$ ), this is consistent with platelet activation taking place during the actual blood sampling.

FIGURE 4.4 URINARY EXCRETION OF Tx-M AND PGI-M IN PATIENTS WITH SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON.



The influence of ex vivo activation may therefore also have contributed to the significantly lower threshold concentration for platelet aggregation in response to arachidonic acid in the patients ( $0.16 \pm 0.04$  vs  $0.38 \pm 0.06$  mM;  $p < 0.005$ ). The capacity of platelets to generate thromboxane in serum was normal in the patients ( $301 \pm 72$  vs  $276 \pm 71$  ng/ml) and all the subjects studied had normal platelet counts.

### Effect of Cooling:

Typical clinical signs of Raynaud's phenomenon developed in the digits of all the patients with systemic sclerosis during cooling. There was a concomitant significant increase ( $p < 0.05$ ) in Tx-M excretion from  $416 \pm 80$  to  $1007 \pm 212$  pg/mg creatinine during cooling (Figure 4.5). There was also a smaller increase in PGI-M excretion from  $248 \pm 39$  to  $362 \pm 81$  pg/mg creatinine (Figure 4.6), but this failed to reach statistical significance. In the healthy control subjects there was no significant effect of cooling on the excretion of either metabolite. Plasma concentrations of B-thromboglobulin and platelet factor 4 were unaltered by cooling in either the patients or controls (Figure 4.7) and the threshold aggregating concentration of arachidonic acid was also unchanged in the patients ( $0.16 \pm 0.04$  mM before vs  $0.21 \pm 0.03$  mM after cooling) and in the control subjects ( $0.38 \pm 0.06$  mM before vs  $0.43 \pm 0.07$  mM after cooling).

FIGURE 4.5 EFFECT OF COOLING ON Tx-M EXCRETION IN PATIENTS WITH SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON.

2,3-DINOR-Tx B<sub>2</sub>  
(pg/mg creatinine)

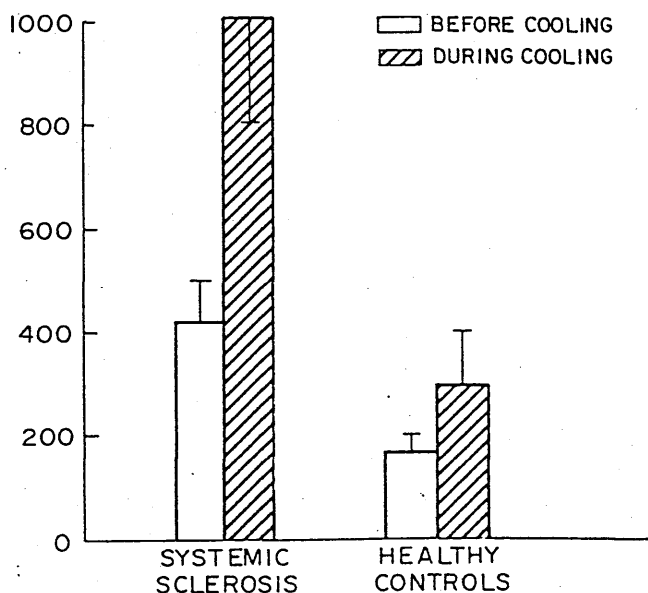


FIGURE 4.6 EFFECT OF COOLING ON PGI-M EXCRETION IN PATIENTS WITH SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON.

2,3-DINOR-6-KETO-PGF<sub>1α</sub>  
(pg/mg creatinine)

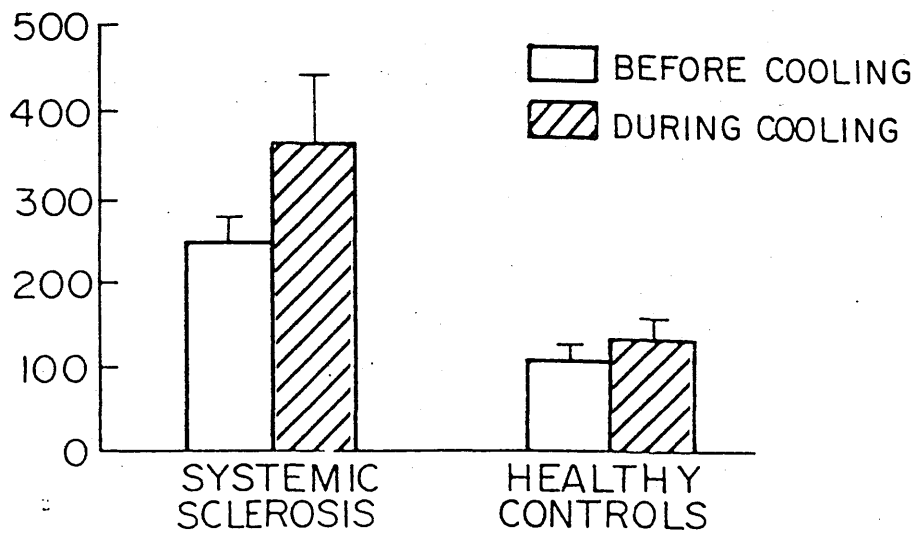
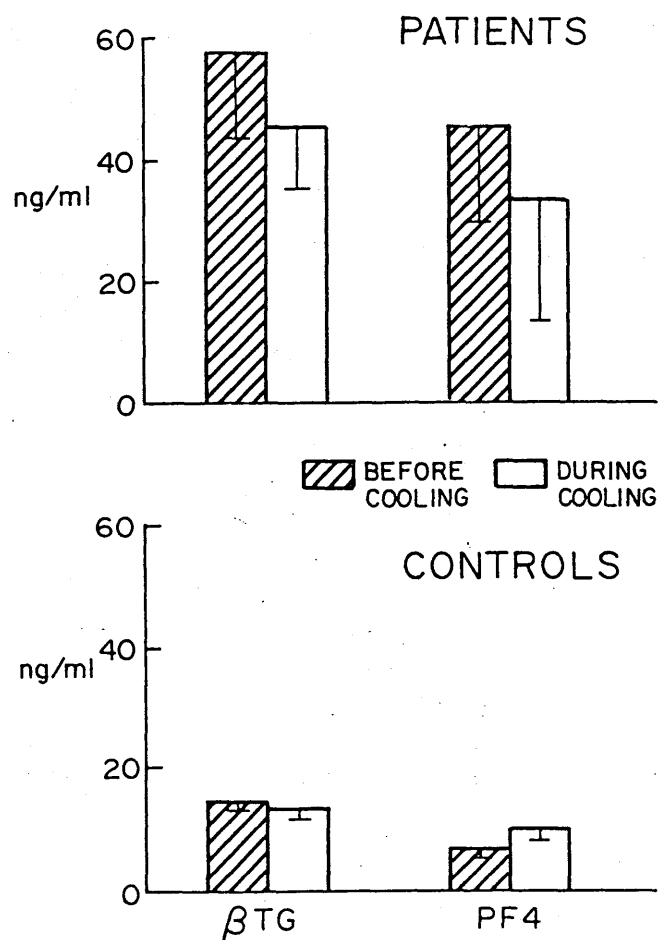




FIGURE 4.7 EFFECT OF COOLING ON PLASMA CONCENTRATIONS OF  $\beta$ -THROMBOGLOBULIN AND PLATELET FACTOR 4 IN PATIENTS WITH SYSTEMIC SCLEROSIS AND RAYNAUD'S PHENOMENON.



## Discussion

This study has shown that in patients with systemic sclerosis complicated by Raynaud's phenomenon endogenous biosynthesis of thromboxane is enhanced. Moreover, a further increase in thromboxane synthesis occurred when the syndrome was precipitated by exposure to cold. Such an observation is consistent with the presence of platelet activation *in vivo* and suggests that in patients with systemic sclerosis, platelets are activated on application of a cold stimulus sufficient to induce Raynaud's phenomenon. The concomitant increase in prostacyclin biosynthesis, as seen in the patients with severe atherosclerosis, supports this hypothesis.

Several previous studies have reported enhanced platelet reactivity in patients with systemic sclerosis and Raynaud's phenomenon. Keenan and Porter demonstrated increased numbers of platelet  $\alpha$ -adrenergic receptors (431) while others have found that platelets from such patients are more responsive to platelet agonists (427)(429). In addition, various studies have demonstrated significant elevations of B-thromboglobulin and the

circulating platelet aggregate ratio (427)(428)(429), as in the present study. However, the coincidental increase in platelet factor 4 seen in this study highlights the difficulty in interpreting indices of platelet activation obtained using invasive sampling procedures. It seems likely that the increase in B-thromboglobulin is at least in part attributable to platelet activation ex vivo during blood sampling, particularly in such a group of patients in which venous access is impaired as a result of the disease process. In this setting, plasma thromboxane levels, although reported to be increased in patients with Raynaud's phenomenon (393), are likely to be extremely misleading and illustrate the advantage of measuring urinary metabolites as a non-invasive approach to investigating eicosanoid biosynthesis (130).

The pathogenesis of systemic sclerosis and of Raynaud's phenomenon is not clearly understood. Platelets have been implicated, both in contributing to acute occlusive episodes (184)(431) and in evolution of the chronic disease process (425)(426)(432). While treatment with platelet inhibitors such as prostacyclin and PGE<sub>1</sub> has been reported to be successful (433)(434)(435), this may reflect the vasodilator activity of these compounds. This study does not directly address the role of platelets in these conditions, but does provide evidence that platelet

activation is occurring and is enhanced on exposure to cold. It therefore identifies a second model of platelet activation in vivo in which thromboxane formation is increased and which represents an appropriate target for therapeutic intervention with regimens designed to inhibit thromboxane synthesis.

### III.MYELOPROLIFERATIVE DISORDERS

It is well recognized that the myeloproliferative disorders are associated with an increased incidence of thrombosis, particularly in the microvascular circulation (436)(437). Qualitative abnormalities of platelet behaviour are common in such patients and may result either in defective platelet function or in enhanced platelet reactivity. Numerous types of abnormality have been described, including altered arachidonate metabolism (195)(196)(438) and several studies have demonstrated evidence of platelet activation in vivo in a variable proportion of patients (439)(440)(441)(442).

The following study investigates endogenous

biosynthesis of thromboxane in a small group of patients with myeloproliferative disorders. The aim was to characterize a further model of platelet activation in vivo and to assess from this preliminary data whether measurement of Tx-M excretion was likely to represent a useful guide to selecting those patients for whom platelet-inhibitory therapy would be beneficial.

### Study Design

#### Patient selection:

Six male patients aged 42-76 years and six healthy age- and sex-matched controls were studied. All of the patients were attending the haematology outpatient clinic of the Veterans Administration Hospital, Nashville, Tennessee. Five of the patients had stable phase chronic granulocytic leukaemia (4 were Philadelphia chromosome-positive, 1 was not tested) and one patient had polycythaemia rubra vera. Diagnoses were made on the basis of bone marrow aspirates, trephine biopsies and neutrophil alkaline phosphatase scores in each case, with additional blood volume studies

in the patient with polycythaemia vera. Although an attempt was made to include further patients with polycythaemia vera and essential thrombocythaemia in the study, almost all of such patients were already on anti-platelet therapy or were unsuitable for study for other reasons (eg renal impairment).

#### Analytical methods:

Endogenous biosynthesis of thromboxane and prostacyclin was determined by measuring Tx-M and PGI-M excretion in urine by gas chromatography mass spectrometry as described in chapter 2. Blood samples corresponding to the time of urine collection were taken for plasma B-thromboglobulin and platelet factor 4 and for serum thromboxane B<sub>2</sub> which were measured by radioimmunoassay. The circulating platelet aggregate ratio was calculated using a modification of the method of Wu and Hoak (350) and the bleeding time was determined by the template method.

#### Results

The results are shown in Table 4.1. Thromboxane

biosynthesis was increased in the patients with myeloproliferative disorders compared to the healthy control subjects (412 +/- 115 vs 208 +/- 43 pg/mg creatinine;  $p < 0.05$ ). Serum thromboxane  $B_2$  generation was also significantly greater ( $p < 0.05$ ) in the patients compared to the healthy controls (394 +/- 47 vs 283 +/- 43 ng/ml). However, unlike the patients with atherosclerosis or systemic sclerosis, the peripheral blood platelet counts were considerably higher in the myeloproliferative patients compared to their controls (543 +/- 84 vs 252 +/- 24  $\times 10^9/l$ ;  $p < 0.05$ ). Since there is a linear relationship between platelet count and both Tx-M and serum thromboxane (see Appendix I), the values of these parameters were corrected to a platelet count of 252  $\times 10^9/l$  to allow direct comparison with the values obtained in the control subjects. This reduced Tx-M excretion in the myeloproliferative patients to 194 +/- 74 pg/mg creatinine and serum thromboxane  $B_2$  to 221 +/- 43 ng/ml, which were not significantly different from the values in the healthy controls.

There was also no difference in PGI-M excretion between patients and controls. Although plasma levels of B-thromboglobulin were increased in the patients (42 +/- 9 vs 10 +/- 3 ng/ml;  $p < 0.05$ ), this was accompanied by a similar elevation of platelet factor 4. The CPA ratio was

**TABLE 4.1 EICOSANOID SYNTHESIS AND PLATELET FUNCTION IN PATIENTS WITH MYELOPROLIFERATIVE DISORDERS.**

	Patients	Controls
Tx-M (pg/mg creatinine)	412±115*	208±43
PGI-M (pg/mg creatinine)	162±43	220±31
Serum thromboxane B <sub>2</sub> (ng/ml)	394±47*	283±43
Platelet count (x 10 <sup>9</sup> /l)	543±84*	252±24
β-thromboglobulin (ng/ml)	42±9*	10±3
Platelet factor 4 (ng/ml)	32±12	11±4
Bleeding time (minutes)	3.5±0.4	3.5±0.4

Values are mean ± SEM of determinations in 6 patients and 6 healthy controls.

\* p <0.05



slightly higher in the myeloproliferative patients (1.11 +/- 0.04 vs 1.01 +/- 0.04) but this difference was not statistically significant. There was also no difference in the bleeding time between the two groups studied.

### Discussion

The recognition that the myeloproliferative syndromes are associated with an increased frequency of thrombotic complications and may exhibit platelet hyperaggregability and platelet activation in vivo (439)(440)(441)(442) suggested that such patients might also have an enhanced rate of thromboxane synthesis. The results of the present study show that although endogenous thromboxane biosynthesis was significantly enhanced in the patients with myeloproliferative disorders, this appears mainly to be a consequence of the higher platelet counts found in the patients. Thus, if the level of Tx-M was adjusted to take account of the higher platelet counts in the patients, there was no longer a significant difference between patients and controls. In addition, in contrast to

the other two models of platelet activation described in this chapter, there was no increase in endogenous prostacyclin formation. Similarly, while plasma B-thromboglobulin was elevated in the patients, the concomitant increase in platelet factor 4 suggests that this was more likely to be due to the higher platelet counts (443) and/or platelet activation occurring during blood sampling (265) rather than the presence of platelet activation in vivo.

There are several reasons for the failure to demonstrate convincing evidence of platelet activation in vivo in this study. Firstly, the numbers studied were small which reflected the difficulty in identifying patients not already on platelet-inhibitory therapy and without other major clinical problems. Secondly, the group of patients studied consisted mainly of individuals with chronic granulocytic leukaemia who are known to have a lower incidence of thrombotic complications than the other myeloproliferative states (436). In addition, none of the patients investigated had a definite history of previous thrombotic episodes and this may therefore have been a low risk population in what is recognized to be a very heterogeneous group of disorders. Finally, the higher platelet counts in these patients made it difficult to accurately interpret the measured indices in terms of the

presence or absence of platelet activation in vivo.

It is likely that clarification of the role of thromboxane in the pathogenesis of the thrombotic complications of the myeloproliferative syndromes will require further studies in selected patients with documented microvascular insufficiency. The results of the present study suggest that as a group the myeloproliferative disorders may not represent a useful model of platelet activation in vivo and underline the importance of identifying appropriate models for the investigation of the mechanisms of human disease.

### CONCLUSION

These studies have shown that in two distinct human syndromes of platelet activation in vivo endogenous biosynthesis of thromboxane is significantly enhanced. Moreover, an additional stimulus to platelet activation (the precipitation of Raynaud's phenomenon on exposure to cold) in the patients with systemic sclerosis resulted in a further increment in thromboxane formation in vivo. The identification of these syndromes of thromboxane-dependent platelet activation provides clinically-relevant models in which to investigate the pharmacology and efficacy of therapeutic regimens directed towards the inhibition of thromboxane synthesis.

The utility of Tx-M as an index of platelet activation in vivo, both in the setting of chronic disease and in response to acute changes, was also confirmed in these studies. Since samples are obtained non-invasively, the values measured are not influenced by ex vivo platelet

activation which can lead to marked artifactual elevations in other parameters such as B-thromboglobulin and plasma thromboxane  $B_2$  (130)(263)(264)(265). The results of a recent study in patients with unstable angina, in whom episodes of ischaemic chest pain were accompanied by phasic increases in Tx-M excretion (422), supports the contention that this approach will be useful in evaluating the role of platelets in the wide variety of conditions in which they have been implicated.

## Chapter 5

### SELECTIVE INHIBITION OF ENDOGENOUS THROMBOXANE FORMATION

#### : Studies in a Human Model of Platelet Activation in vivo

## INTRODUCTION

### I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE:

#### Study Design:

Patient selection

Protocol

Clinical evaluation

Analytical methods

#### Results:

Evidence of platelet activation in vivo

Effects on thromboxane synthesis in vivo and ex vivo

Effects on prostacyclin biosynthesis

Effects on platelet function

Plasma drug levels

Clinical evaluation

#### Discussion

## II. INHIBITION OF THROMBOXANE BIOSYNTHESIS BY FISH-OIL ADMINISTRATION:

### Study Design:

Patient selection

Protocol

Analytical methods

### Results:

Evidence of platelet activation in vivo

Effects on thromboxane synthesis in vivo and ex vivo

Effects on prostacyclin biosynthesis

Effects on platelet function

Effects on erythrocyte fatty acids

Clinical Evaluation

Discussion

CONCLUSION



## INTRODUCTION

Platelet-inhibitory therapy has been advocated for the treatment and prevention of a wide spectrum of diseases in which platelet activation is believed to play a role. Aspirin was the first drug to be evaluated in large clinical studies and appeared to be of some benefit, particularly in cerebrovascular and cardiovascular atherosclerosis

(229-232)(267-270)(272)(273)(362)(444)(445). Nevertheless, the results of individual controlled trials in these conditions were much less dramatic than had been anticipated on the basis of aspirin's ability in vitro to inhibit platelet function and thromboxane synthesis (446). While there are clearly many reasons for this, the recognition that aspirin also caused a significant reduction in the synthesis of a potent endogenous inhibitor of platelet function, prostacyclin, stimulated the search for pharmacological agents which selectively inhibited thromboxane formation.

Several approaches to selective inhibition of

thromboxane synthesis were investigated in healthy individuals in chapter 3. However, the effects of platelet-inhibitory therapy in patients with platelet activation in vivo would be expected to be quite different. For example, in normal healthy subjects the rate of endogenous thromboxane biosynthesis is extremely low (92) and, as I have shown, a single dose of aspirin will significantly reduce it. In the presence of in vivo platelet activation when thromboxane formation is increased, it may consequently be more difficult to inhibit endogenous thromboxane production. In addition, since the platelet capacity for thromboxane generation greatly exceeds the actual biosynthetic rates even in the presence of platelet activation in vivo, it is likely that in these patients inhibition of thromboxane synthesis will have to be virtually complete before thromboxane-dependent platelet aggregation is abolished. Furthermore, prostacyclin biosynthesis is also increased in such patients. Since prostacyclin not only inhibits the aggregation of platelets by all recognized agonists, but is also a potent vasodilator and disaggregates previously aggregated platelets (15), it may function as an important regulator of platelet-vascular homeostasis in the presence of vessel narrowing or occlusion, lending further support to the argument in favour of selective regimens. For these reasons, the potential therapeutic benefit of such

regimens needs to be evaluated by investigating their functional and biochemical effects in diseases in which platelet activation in vivo and associated enhanced biosynthesis of thromboxane have been shown to occur.

The following studies have been carried out in patients with severe atherosclerosis who had evidence of platelet activation in vivo and increased thromboxane biosynthesis. The patients were chosen, on the basis of the work described in chapter 4, to represent a stable model of thromboxane-dependent platelet activation in vivo which could be evaluated over a period of months. The effects of two different methods of inhibiting endogenous thromboxane formation have been investigated- a direct approach employing specific blockade of thromboxane synthase, and an indirect approach in which chronic administration of fish-oil was used to inhibit platelet activation and consequently reduce reduce thromboxane biosynthesis. Thromboxane synthase inhibitors are the only drugs known to enhance endogenous biosynthesis of prostacyclin and may therefore offer a particular advantage in the setting of vaso-occlusive disease. Dietary supplementation with fish-oils rich in eicosapentaenoic acid has been shown in healthy individuals to diminish the capacity of platelets

to form thromboxane  $B_2$  (316)(317)(319) and to inhibit platelet function (316)(317)(322)(321)(447)(448). In addition, preliminary evidence suggests that prostacyclin biosynthesis is preserved and that prostaglandin  $I_3$ , which has similar biological properties to prostacyclin, is formed (449) and may contribute to the anti-platelet effects of this approach.

#### I. SELECTIVE INHIBITION OF THROMBOXANE SYNTHASE

Although, they appear to have relatively weak anti-platelet activity in vitro, there is considerable evidence that thromboxane synthase inhibitors can significantly reduce mortality and morbidity in several animal models of thrombosis (202)(228) and vascular occlusion (203-206). Dazoxiben has been shown to eliminate or attenuate cyclical flow reductions in stenotic canine coronary arteries (206) and in another canine model to delay the time for occlusive thrombi to form at sites of electrical injury in coronary vessels (205). Furthermore, in the studies of Aiken et al (203) and Fitzgerald et al (345) the efficacy of thromboxane synthase inhibitors in this setting appeared to be due at least in part to the

ability of these drugs to enhance prostacyclin formation.

Platelet granule constituents, including platelet-derived growth factor, 5-hydroxytryptamine, the adenine nucleotides and platelet activating factor, released when platelets are activated are potent stimuli for prostacyclin formation (114)(116)(117)(118). The observation that basal prostacyclin biosynthesis is significantly higher in patients with platelet activation in vivo, raises the possibility that transfer of platelet-derived prostaglandin endoperoxides to prostacyclin synthase might be facilitated in such patients. Thus, the potential of thromboxane synthase inhibitors to increase prostacyclin biosynthesis may be even greater. Nevertheless, these drugs have never been evaluated in a human model of vaso-occlusive disease in which documented evidence of thromboxane-dependent platelet activation in vivo provides the rationale for such an approach. Previous clinical studies have generally yielded very disappointing results (379). However, the studies have largely been confined to heterogeneous groups of patients in which the role of thromboxane has not been clearly defined and the effects on thromboxane and prostacyclin biosynthesis have not been assessed.

This study examines the biochemical and functional

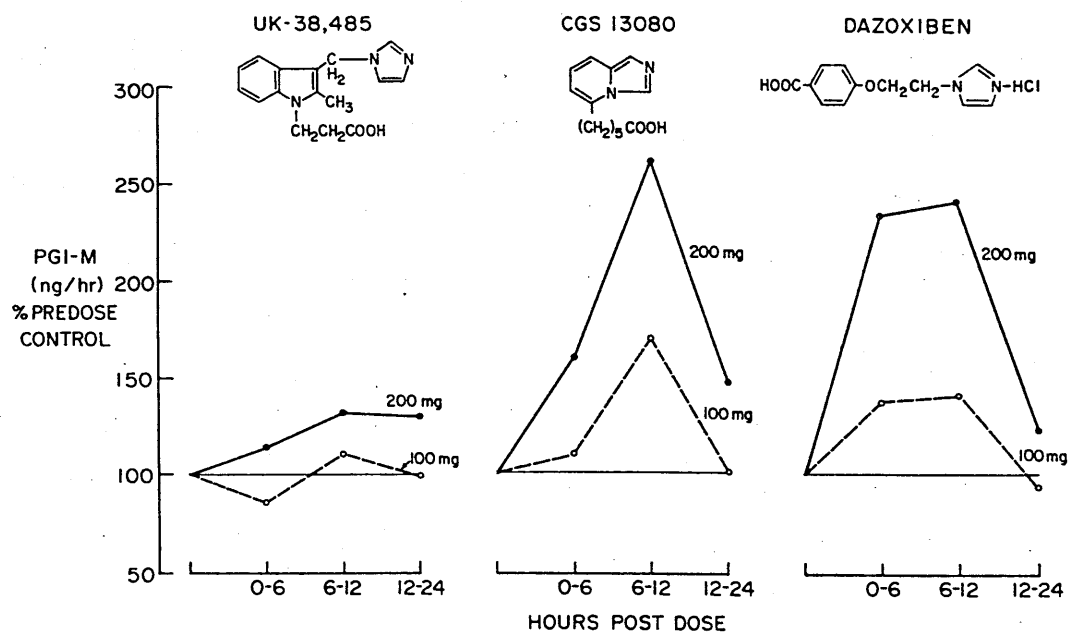
effects of thromboxane synthase inhibition in a human model of platelet activation. The aim was to determine whether platelet activation persisted during chronic administration of these drugs and whether prostacyclin formation was further enhanced. The thromboxane synthase inhibitor selected for the study was imidazo (1,5-2)pyridine-5-hexanoic acid (CGS 13080) which, in healthy individuals, induces a greater increase in prostacyclin biosynthesis than the other two compounds of this class on which data are available (Figure 5.1).

#### STUDY DESIGN:

##### Patient selection:

Nine patients (8 men and one woman, aged 49-75 years) with angiographically-confirmed peripheral arterial disease of the lower limb were studied. The patients had severe generalized disease (grade III or IV) with pain at rest and/or ischaemic ulcers. Each patient fulfilled predetermined criteria consistent with the presence of platelet activation in vivo and are described in detail in chapter 4. The criteria for inclusion were a plasma

FIGURE 5.1 PGI-M EXCRETION AFTER ORAL ADMINISTRATION OF VARIOUS THROMBOXANE SYNTHASE INHIBITORS.



B-thromboglobulin concentration of > 20ng/ml (in association with a normal platelet factor 4 level of < 18 ng/ml) and a circulating platelet aggregate (CPA) ratio of more than or equal to 1.3. In addition, all the patients had increased excretion of the major urinary metabolites of prostacyclin (PGI-M) and thromboxane (Tx-M) and a shortened bleeding time. Of the nine patients, five were insulin-dependent diabetics, seven had electrocardiographic evidence of chronic myocardial ischaemia and seven were cigarette smokers. Four of the patients had foot ulcers at the beginning of the study and one further patient developed an ulcer during the course of the study.

#### Protocol:

The study was randomized, double-blind and placebo-controlled. The patients received either CGS 13080 100mg, CGS 13080 200mg or two matching placebo capsules as a single dose followed 24 hours later by a regimen of one dose every 6 hours for 6 days. The drug and placebo capsules were kindly provided by Dr Nancy Feliciano of the Ciba-Geigy Corporation, Summit, New Jersey. The order of the three treatment regimens was randomized according to a crossover design, each of the treatments being separated



by 7-day drug-free intervals so that each patient was studied over a period of approximately 6 weeks. All of the patients refrained from taking aspirin or other non-steroidal anti-inflammatory drugs during and for at least 10 days prior to the commencement of the study.

After an overnight fast and cessation of cigarette smoking for a minimum of 30 minutes, blood was drawn from a fresh venepuncture for determination of serum thromboxane  $B_2$ , plasma B-thromboglobulin, platelet factor 4 and the CPA ratio before and 1,4,6,8 and 24 hours after the single dose and before and after the final dose of each 6-day regimen. Blood was collected for platelet aggregation studies and for the measurement of plasma drug levels at the same times and for full blood counts and a routine automated chemistry screen before dosing on each of the study days of the three treatment periods. The bleeding time was estimated before and one hour after the first and last dose of each treatment. Twenty-four hour urine collections for measurement of the major urinary metabolites of thromboxane (Tx-M) and prostacyclin (PGI-M) were made before and in three aliquots after (0-6, 6-12 and 12-24 hours) the single dose and the final dose of the chronic dosing period.

On a separate occasion four of the patients were

administered 325mg aspirin as a single dose. Blood was drawn for measurement of serum thromboxane B<sub>2</sub> and platelet aggregation in response to arachidonic acid, collagen and adrenaline before and one hour after dosing.

#### Clinical evaluation:

Before the study and at the end of each treatment period, the patients were assessed clinically by physical examination and objectively by Doppler flow measurements of ankle systolic pressure, by analysis of the waveform of arterial flow and by calculation of the ankle/brachial systolic pressure index (450). Measurements were made in patients at rest with a unidirectional Doppler ultrasound velocity detector (Park Electronics, Aloha, Oregon) and a mercury sphygmomanometer. A visual analogue scale was used to monitor pain at rest. This consisted of a 10cm line labelled "no pain" and "the worst pain I have ever experienced" at opposite poles.

#### Analytical methods:

The presence of platelet activation was determined by measuring plasma B-thromboglobulin and platelet factor 4

by radioimmunoassay as described in chapter 2 and the CPA ratio by a modification of the method of Wu and Hoak (350). In addition, Tx-M and PGI-M were quantitated by stable isotope dilution assays employing gas chromatography/mass spectrometry. Urinary thromboxane  $B_2$  was also measured by a stable isotope dilution assay, as described in chapter 2, using gas chromatography/mass spectrometry and thromboxane generation in serum was determined by radioimmunoassay. Platelet function was assessed by measuring the bleeding time by the template method and by platelet aggregation studies in PRP in response to arachidonic acid (0.33mM and 1.33mM), collagen (1.9 ug/ml and 19 ug/ml) and adrenaline (5 and 10uM) and in whole blood in response to arachidonic acid (0.66mM) and collagen (9.5 ug/ml). Plasma concentrations of CGS 13080 were determined by Dr Chan of the Ciba-Geigy Corporation using high-pressure liquid chromatography.

## RESULTS:

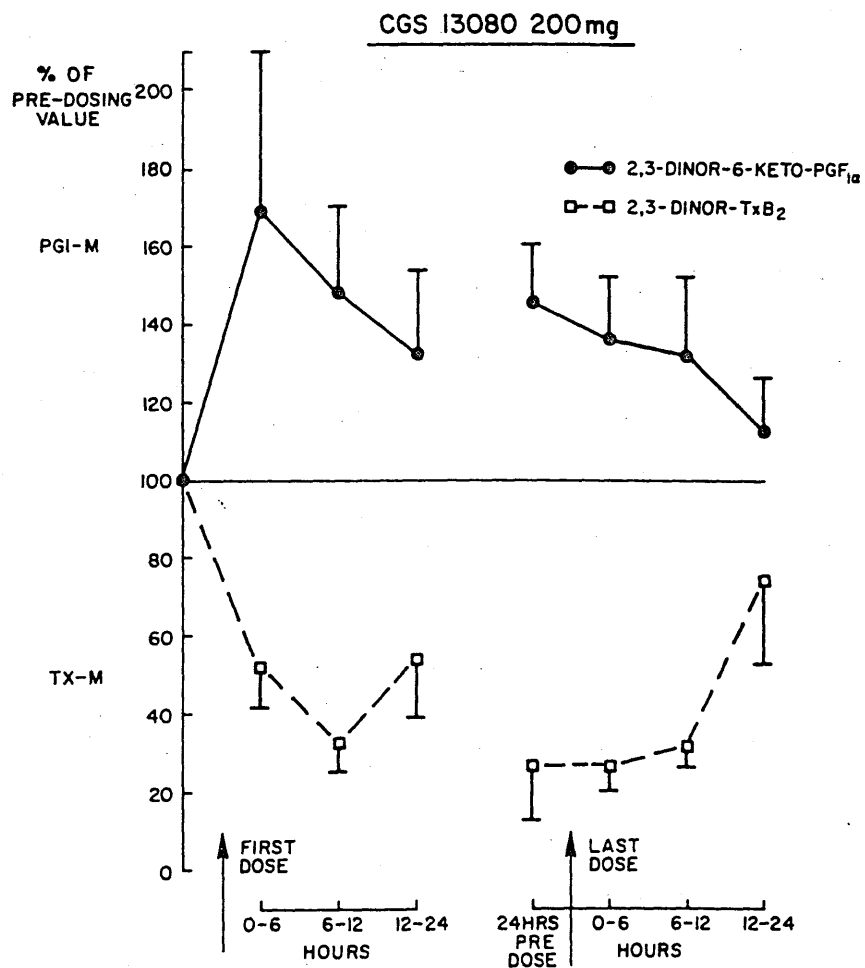
### Evidence of Platelet Activation in vivo:

The patients all fulfilled the preset criteria consistent with platelet activation in vivo. Plasma B-thromboglobulin was elevated at  $113 \pm 22$  ng/ml with normal concentrations of platelet factor 4 ( $12 \pm 0.5$  ng/ml). The CPA ratio was increased at  $1.4 \pm 0.01$  and the bleeding time was shortened at  $2.9 \pm 0.2$  minutes. Urinary excretion of both Tx-M and PGI-M was enhanced at  $912 \pm 99$  and  $323 \pm 89$  pg/mg creatinine respectively.

### Effects on Thromboxane Synthesis in vivo and ex vivo:

Tx-M excretion was unaltered after administration of placebo and did not differ significantly on the control days that preceded each treatment period. After administration of a single dose, CGS 13080 caused a significant, reversible fall in Tx-M by a maximum of  $66 \pm 4$  % from  $716 \pm 213$  to  $240 \pm 81$  pg/mg creatinine 6-12 hours after dosing (Figure 5.2). Although Tx-M excretion remained depressed during chronic dosing,

FIGURE 5.2 EXCRETION OF TX-M AND PGI-M AFTER ADMINISTRATION OF CGS 13080 TO PATIENTS WITH ATHEROSCLEROSIS.



significant biosynthesis of thromboxane persisted and no further decrement in Tx-M occurred with administration of the final dose. There was no significant difference between the 100mg and 200mg dose with respect to the effects observed on Tx-M excretion after short- and long-term dosing (Table 5.1). In the four patients in whom the urinary excretion of both thromboxane  $B_2$  and Tx-M was measured, a similar degree of depression of both metabolites was noted (Table 5.2). In addition, basal levels of urinary thromboxane  $B_2$  were markedly elevated compared with those observed in healthy subjects ( $247 \pm 86$  vs  $46 \pm 14$  pg/mg creatinine).

Administration of placebo did not significantly alter serum thromboxane  $B_2$  levels from predosing values. After single-dose administration of CGS 13080, serum thromboxane  $B_2$  generation ex vivo was reversibly inhibited, with peak inhibition measured one hour after dosing (Figure 5.3). Recovery of serum thromboxane was linear and gave a calculated "biological" half-life (50% recovery to predosing values) of between 6 and 8 hours. The level of serum thromboxane  $B_2$  immediately before the last dose ( $140 \pm 54$  ng/ml), which was measured 8 hours after the previous dose, was not significantly different from that measured after the first dose ( $115 \pm 38$  ng/ml). Thus, no cumulative inhibition of serum

TABLE 5.1 Tx-M EXCRETION AFTER ADMINISTRATION OF CGS 13080 TO PATIENTS WITH ATHEROSCLEROSIS.

	CGS 13080 100mg	CGS 13080 200mg
Prior to first dose:	737±271	716±213
After first dose		
0-6 hours:	460±224	351±143*
6-12 hours:	390±212*	240±81**
12-24 hours:	441±197*	458±158*
Prior to last dose:	213±109**	106±32**
After last dose		
0-6 hours:	234±99**	152±53**
6-12 hours:	245±117**	195±59**
12-24 hours:	379±147*	372±79*

Values are expressed in pg/mg creatinine (mean ± SEM) in samples from 9 patients.

\* p < 0.05

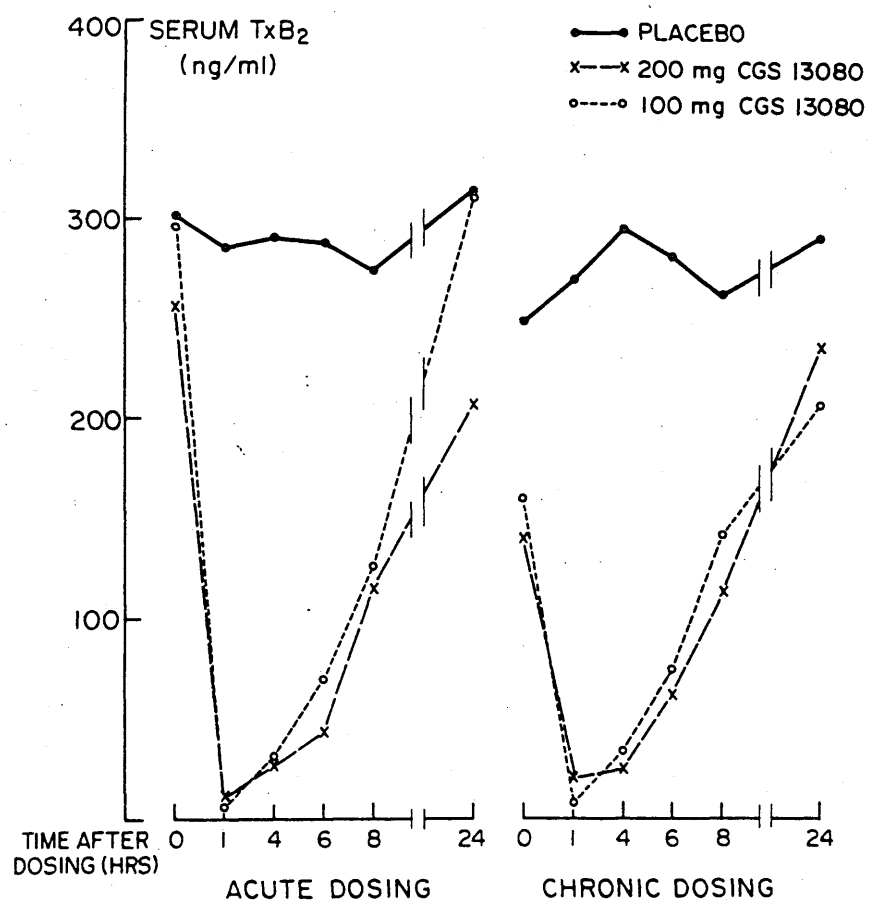
\*\* p < 0.01

TABLE 5.2 URINARY EXCRETION OF Tx-M AND THROMBOXANE B<sub>2</sub> (TxB<sub>2</sub>) after CHRONIC ADMINISTRATION OF CGS 13080 200mg TO PATIENTS WITH ATHEROSCLEROSIS.

	% inhibition from predose control	
	Tx-M	TxB <sub>2</sub>
Prior to last dose:	84.7±5	89.4±1
After last dose		
0-6 hours:	78.0±6	88.2±7
6-12 hours:	71.6±7	72.7±4
12-24 hours:	52.1±12	52.3±7

Values are mean ± SEM of determinations in 4 patients.

FIGURE 5.3 EFFECT OF CGS 13080 ON SERUM THROMBOXANE B<sub>2</sub> GENERATION IN PATIENTS WITH ATHEROSCLEROSIS.





thromboxane occurred during chronic dosing. As observed with Tx-M excretion, there was no difference in the effects of the two doses of CGS 13080 on serum thromboxane B<sub>2</sub> formation, suggesting their proximity on the dose-response curve.

#### Effect on Prostacyclin Biosynthesis:

There was no change in PGI-M excretion with placebo. Coincident with the fall in Tx-M after 200mg of CGS 13080, PGI-M excretion increased significantly ( $p < 0.05$ ) from 291 +/- 54 to 538 +/- 150 and 442 +/- 150 pg/mg creatinine in the 0-6 and 6-12 hour aliquots. Similar increments in PGI-M were obtained after the 100mg dose (Table 5.3). PGI-M remained significantly elevated during chronic dosing but no further increase occurred after the final dose of CGS 13080 (Figure 5.2).

#### Effects on Platelet Function:

Plasma B-thromboglobulin and platelet factor 4 were unchanged by placebo. There was no significant depression of B-thromboglobulin after single-dose administration of either dose of CGS 13080 (Figure 5.4). During chronic

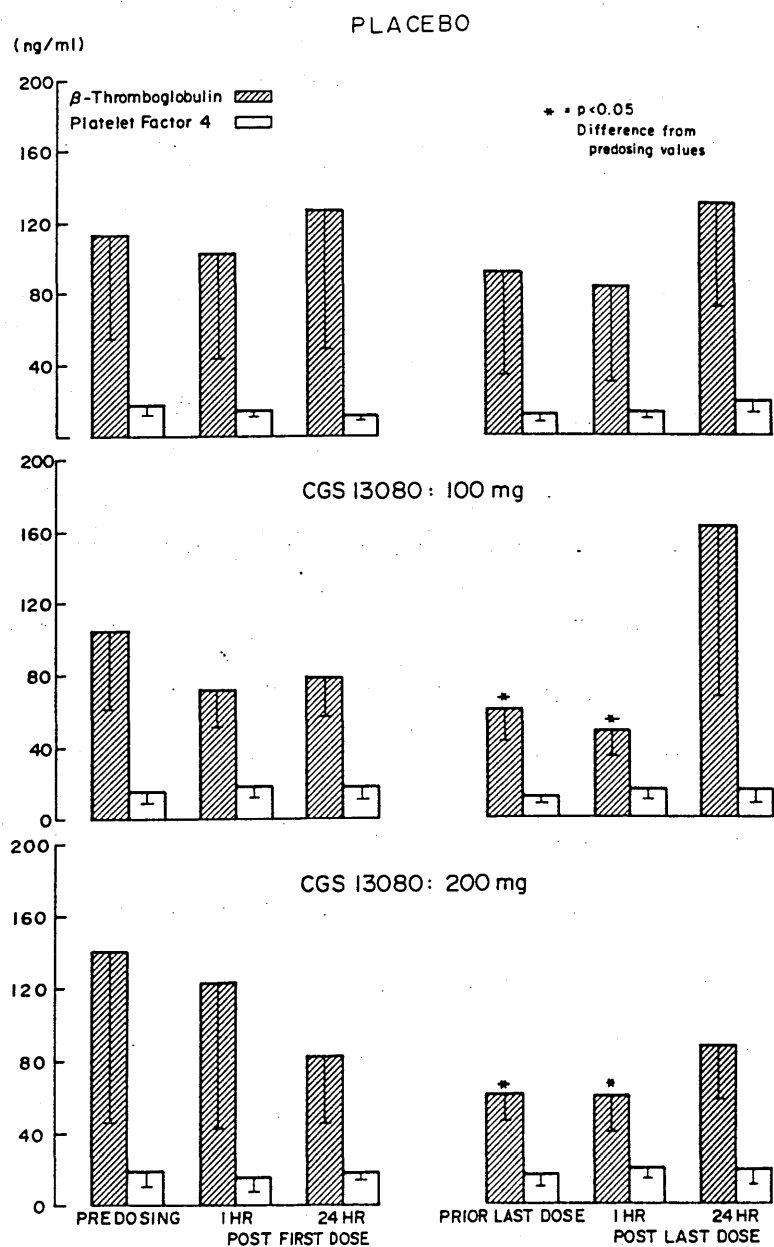
**TABLE 5.3 PGI-M EXCRETION AFTER ADMINISTRATION OF CGS 13080 TO PATIENTS WITH ATHEROSCLEROSIS.**

	CGS 13080 100mg	CGS 13080 200mg
Prior to first dose:	287±24	291±54
After first dose		
0-6 hours:	444±110*	538±150*
6-12 hours:	441±148*	442±150*
12-24 hours:	402±151	399±139
Prior to last dose:	367±66*	419±105*
After last dose		
0-6 hours:	494±92*	364±63
6-12 hours:	406±62*	353±77
12-24 hours:	371±67	334±66

Values are expressed in pg/mg creatinine (mean ± SEM) in samples from 9 patients.

\* p <0.05

FIGURE 5.4 PLASMA CONCENTRATIONS OF B-THROMBOGLOBULIN AND PLATELET FACTOR 4 AFTER ADMINISTRATION OF CGS 13080 AND PLACEBO TO PATIENTS WITH ATHEROSCLEROSIS.



dosing, however, B-thromboglobulin levels fell significantly, although they remained more than three times normal: 61 +/- 21 ng/ml (200mg) and 45 +/- 15 ng/ml (100mg) vs 13 +/- 3 ng/ml (age-matched control subjects). Platelet factor 4 levels were within the normal range and were unaltered by CGS 13080 (Figure 5.4).

The CPA ratio (Figure 5.5) and bleeding time (Figure 5.6) were not significantly different before and after administration of CGS 13080 or placebo. There was no change in platelet aggregation in PRP in response to arachidonic acid (Figure 5.7), adrenaline and collagen (Figure 5.8). Platelet aggregation in whole blood was also unaltered. By contrast, administration of aspirin significantly inhibited platelet function in these patients (Table 5.4).

#### Plasma Drug Levels:

Plasma concentrations of CGS 13080 are shown in Table 5.5. These confirmed bioavailability of the compound and the relationship of plasma concentration to dose within each of the patients.

FIGURE 5.5 EFFECT OF CGS 13080 ON THE CIRCULATING PLATELET AGGREGATE RATIO IN PATIENTS WITH ATHEROSCLEROSIS.

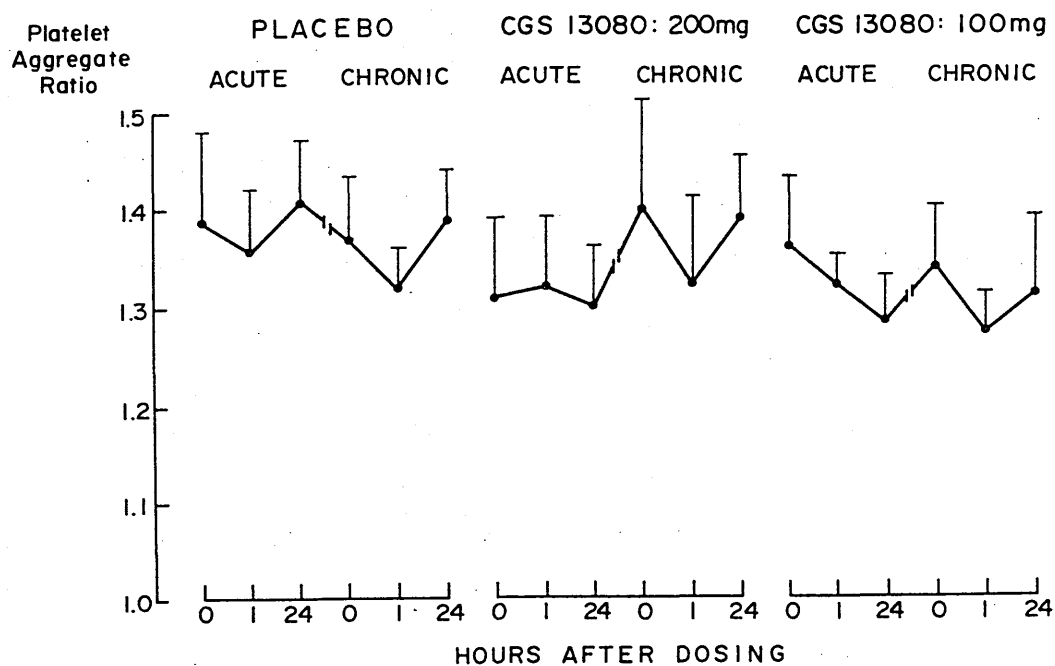


FIGURE 5.6 EFFECT OF CGS 13080 ON THE BLEEDING TIME IN PATIENTS WITH ATHEROSCLEROSIS.

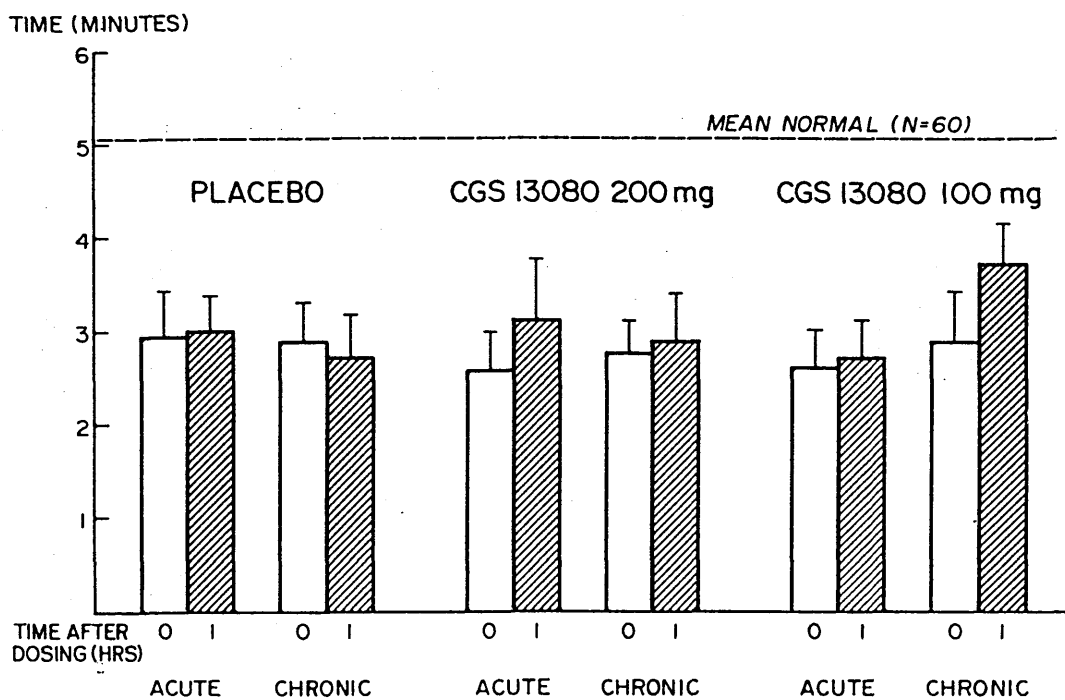


FIGURE 5.7 EFFECT OF CGS 13080 ON THE BLEEDING TIME IN PATIENTS WITH ATHEROSCLEROSIS.

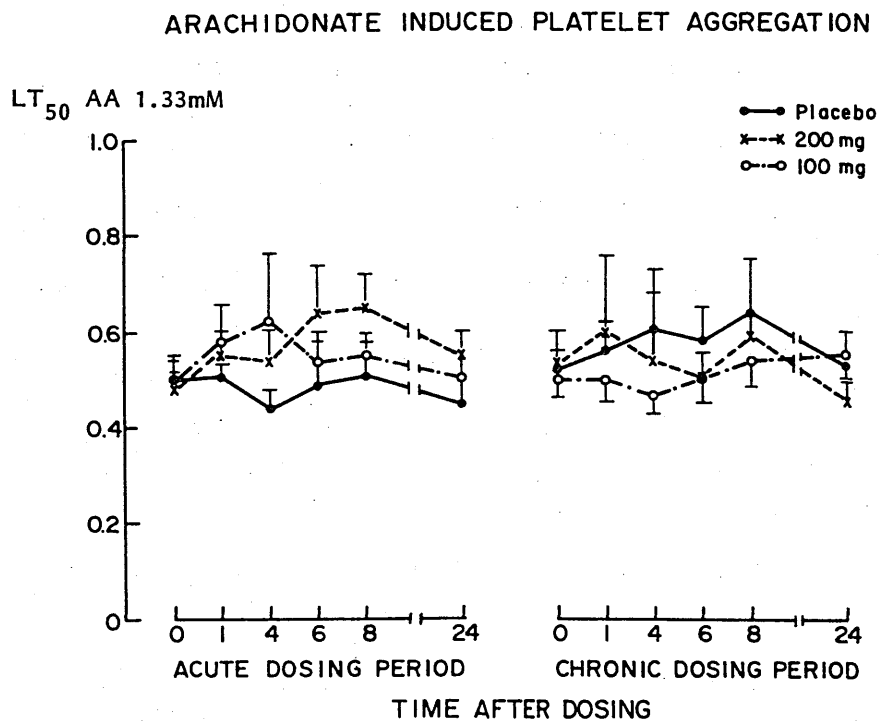


FIGURE 5.8 PLATELET AGGREGATION IN RESPONSE TO COLLAGEN AFTER ADMINISTRATION OF CGS 13080 TO PATIENTS WITH ATHEROSCLEROSIS.

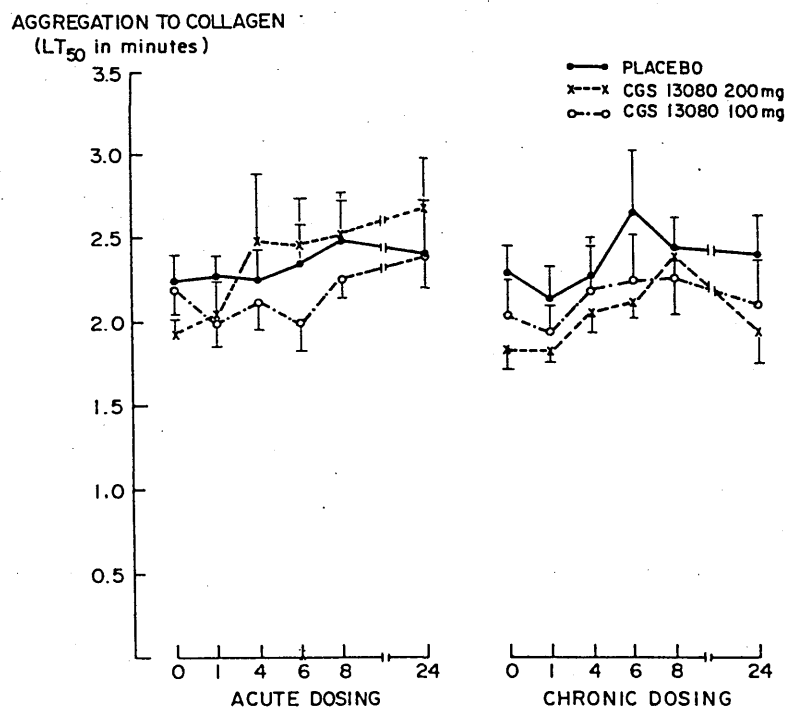


TABLE 5.4 EFFECT OF ASPIRIN 325 mg ON PLATELET AGGREGATION  
BLEEDING TIME AND SERUM THROMBOXANE B<sub>2</sub> IN PATIENTS WITH  
ATHEROSCLEROSIS.

	Before aspirin	After aspirin
Platelet aggregation* in response to:		
Arachidonic acid 1.33mM	87±1.5	NA
0.33mM	87±0.9	NA
Collagen 19µg/ml	92±2	85±4
1.9µg/ml	61±20	NA
Adrenaline 10µM	88±2.5	PA
5µM	89±0.8	PA
Serum thromboxane B <sub>2</sub> (ng/ml)	332±62	13±7
Bleeding time (minutes)	3.6±0.6	5.1±0.8

\* Platelet aggregation in PRP is expressed as a percentage of  
maximal aggregation 6 minutes after addition of the agonist.

Values are mean ± SEM of determinations in 4 patients.

NA : no aggregation

PA : primary wave of aggregation

TABLE 5.5 PLASMA CONCENTRATIONS OF CGS 13080(ng/ml) AFTER SINGLE DOSE AND CHRONIC ADMINISTRATION TO PATIENTS WITH ATHEROSCLEROSIS.

Time after dosing (hours)	CGS 13080 100mg		CGS 13080 200mg	
	First Dose	Last dose of 100mg qid regimen	First Dose	Last dose of 200mg qid regimen
0	ND	53±26	ND	260±213
1	2333±279	1704±397	2476±593	3010±590
4	149±36	128±30	299±122	437±111
6	31±8	49±19	98±53	102±33
8	ND	21±9	20±9	64±23
24	ND	ND	ND	ND

Significant concentrations of CGS 13080 were not detectable in plasma 8 hours after single-dose administration and in eight of the nine patients there was no evidence of accumulation during administration of multiple doses. One patient did, however, exhibit marked accumulation of the drug with long-term dosing, particularly at the higher dose, and this correlated with a greater degree of inhibition of serum thromboxane B<sub>2</sub>, Tx-M excretion and plasma B-thromboglobulin (Figure 5.9) than observed in the other patients.

#### Clinical Evaluation:

There was no significant alteration in the Doppler measurements of absolute ankle systolic pressure, the arterial wave form or the ankle/brachial systolic pressure index (Figure 5.10). The scores measured on the visual



FIGURE 5.9 RELATIONSHIP BETWEEN PLASMA CONCENTRATIONS OF CGS 13080 AND THE EFFECTS ON INDICES OF THROMBOXANE SYNTHESIS AND PLATELET FUNCTION.

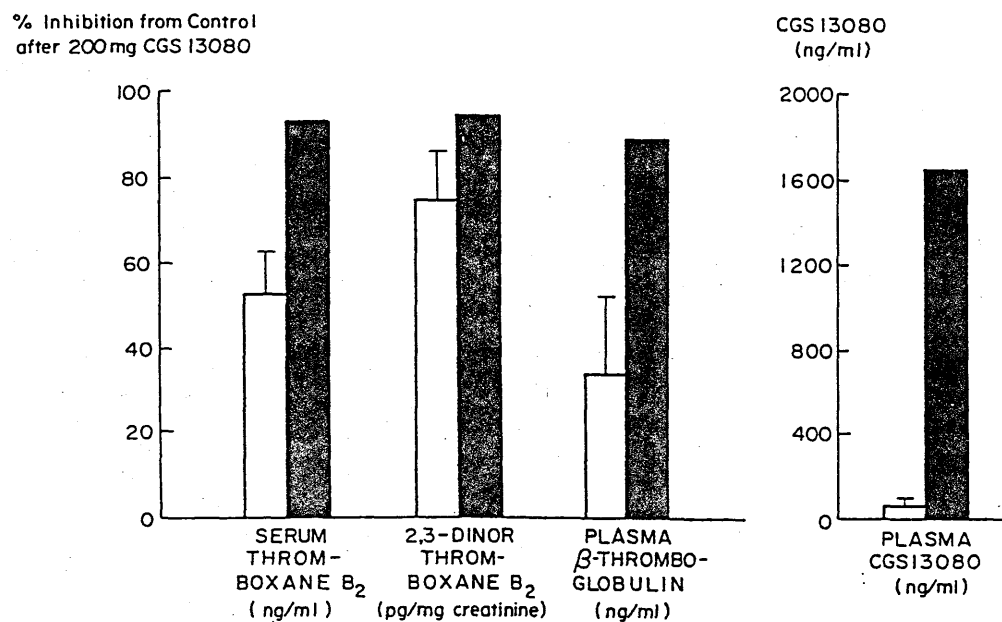
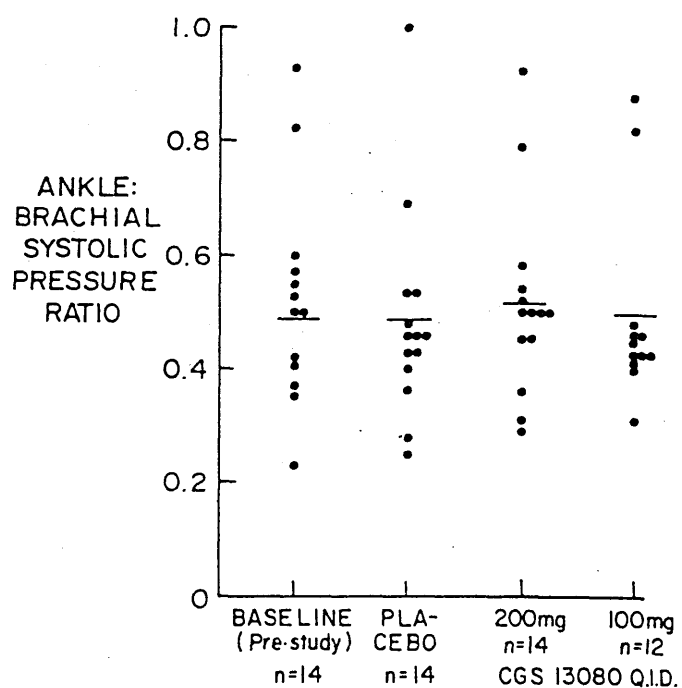


FIGURE 5.10 EFFECT OF CHRONIC ADMINISTRATION OF CGS 13080 ON THE ANKLE : BRACHIAL SYSTOLIC PRESSURE RATIO IN PATIENTS WITH SEVERE ATHEROSCLEROSIS PERIPHERAL VASCULAR DISEASE.



analogue scale used to assess pain at rest were not significantly different with placebo or CGS 13080. At the beginning of the study the routine biochemistry screen and full blood counts, including the platelet counts (mean  $268 \pm 17 \times 10^9/l$ ), of all the patients were within normal limits and did not change significantly during the study.

### Discussion

This study was designed to investigate the biochemical and functional effects of administration of a representative thromboxane synthase inhibitor in patients with platelet activation in vivo and increased endogenous thromboxane biosynthesis. Single-dose administration of CGS 13080 resulted in more than 95% inhibition of serum thromboxane formation ex vivo one hour after dosing, with a biological half-life of 6-8 hours, which is comparable to that achieved with other thromboxane synthase inhibitors (64)(366) and with this compound in healthy volunteers (65)(365). The corresponding reduction in Tx-M excretion after a single dose was 66%. Thus, the thromboxane synthase inhibitor caused a considerably greater fall in Tx-M in the patients with platelet

activation in vivo than that seen in healthy individuals (see chapter 3). During chronic dosing, however, trough levels of serum thromboxane B<sub>2</sub> and Tx-M remained at approximately 50% and 25% respectively of predosing values. Since even a 10% residual capacity to generate thromboxane appears to be enough to fully sustain thromboxane-dependent platelet function in vitro (384), the degree of inhibition of Tx-M achieved in this study would be unlikely to abolish platelet activation in these patients.

Using a variety of methods to characterize platelet function ex vivo and in vivo, the study has clearly demonstrated that platelet activation does persist in this population during chronic administration of a thromboxane synthase inhibitor. There was also no objective evidence of clinical improvement during the study. There was no change in the bleeding time, in platelet aggregation in response to three different agonists or in the CPA ratio, and although B-thromboglobulin fell, it still remained more than three times normal. This is in contrast to studies in healthy volunteers in whom CGS 13080 significantly prolonged the bleeding time and inhibited platelet aggregation ex vivo (65). Nevertheless, that platelet activation was reduced to a significant degree is suggested both by the fall in B-thromboglobulin and by the

disproportionately greater depression of Tx-M excretion induced by the thromboxane synthase inhibitor compared to that seen in healthy individuals without evidence of platelet activation in vivo.

The failure to induce complete, cumulative inhibition of thromboxane generation during chronic drug administration may have several explanations. Firstly, the dosing interval in this investigation, as in previous clinical studies with this class of compounds (393)(394)(451), was based, not on the plasma drug half-life of 45 minutes (365), but on the much longer biological half-life. While cumulation to a steady-state effect would be expected in three to four half-lives when the dosing interval is based on the plasma drug half-life, this assumption cannot be made for the biological half-life. Indeed, 6 to 8 hours after dosing, plasma levels of CGS 13080 were barely detectable and it is thus not surprising that cumulative inhibition of thromboxane formation did not occur during this study. While such a discrepancy (between the biological and pharmacological half-lives) might be anticipated on the basis of established plasma drug kinetic-dynamic relationships (452), other factors, such as the presence of active

metabolites or slow elimination of the drug from deep compartments, may have contributed to this observation. However, the same discrepancy can be seen with several structurally distinct thromboxane synthase inhibitors (64)(65)(366). In no case have biologically active metabolites of these compounds been identified. This suggests that incomplete inhibition of thromboxane biosynthesis during the dosing interval is likely to have occurred in clinical trials of synthase inhibitors other than CGS 13080 since none have employed a dosing interval of less than 6 hours.

Other factors which may contribute to the persistence of platelet activation in vivo in these patients are the effects of accumulated prostaglandin endoperoxides and platelet activation secondary to non-eicosanoid dependent mechanisms. Aspirin, which reduces formation of both thromboxane and the prostaglandin endoperoxides, significantly prolonged the bleeding time in these patients and inhibited platelet aggregation. This observation, and the lack of effect of the synthase inhibitor on measured indices of platelet function one hour after dosing when inhibition of platelet thromboxane formation ex vivo was more than 95%, suggest that substitution for the proaggregatory effects of thromboxane  $A_2$  by accumulated endoperoxides may play a role

during inhibition of thromboxane synthase in vivo. Support for this hypothesis is provided by a canine model in which the combination of an endoperoxide/thromboxane  $A_2$  receptor antagonist and a thromboxane synthase inhibitor delays the time to coronary occlusion despite maximal inhibition of platelet thromboxane synthesis by the synthase inhibitor alone (345). However, that incomplete inhibition of thromboxane formation throughout the chronic dosing period is likely to be a major factor in the continuing platelet activation observed in this study is supported by the data from the single patient in whom significant drug accumulation occurred. In this patient, cumulative inhibition of thromboxane formation in vivo and ex vivo was virtually complete, coincident with a significantly greater fall in B-thromboglobulin.

It is of interest that the pattern of inhibition and recovery of urinary thromboxane  $B_2$  was almost identical to that for the dinor metabolite, Tx-M. In contrast to Tx-M which is mainly derived from platelets, urinary thromboxane  $B_2$  is believed to reflect primarily renal thromboxane formation under physiological conditions (338). Data from a rat glomerular preparation in vitro has shown that glomerular thromboxane synthase appears to be considerably more sensitive to the effects of the synthase inhibitor, dazoxiben, than platelet

thromboxane synthase (453). However, the similar degree of inhibition of urinary thromboxane  $B_2$  and Tx-M seen in the present study suggest that this is unlikely to be true in humans. An alternative explanation of these findings is that in the setting of platelet activation in vivo, unlike in healthy individuals, a significant proportion of urinary thromboxane  $B_2$  may be derived from enhanced platelet thromboxane synthesis. Indeed, basal levels of urinary thromboxane  $B_2$  were elevated in the patients in this study to a similar extent to those of Tx-M. The matching pattern of inhibition and recovery of these two metabolites is also consistent with this interpretation.

Endogenous biosynthesis of prostacyclin increased by approximately two-fold in response to a single dose of the thromboxane synthase inhibitor, which is consistent with endoperoxide redirection to a source of prostacyclin synthase (62)(63). The initial significant increment in prostacyclin appeared not to be maintained during chronic dosing and there was no further increase after the final dose. These changes mirrored the fall in endogenous thromboxane formation and provide additional evidence that alterations in prostacyclin formation are dependent upon increased provision of endoperoxide substrate after inhibition of thromboxane synthase. While the percentage

increment in prostacyclin biosynthesis was similar to that observed with the same drug in healthy individuals (65), the absolute increment in metabolite excretion was at least twice as great in the patients. This suggests that transfer of prostaglandin endoperoxides is enhanced in the presence of platelet activation in vivo. Despite elevated basal excretion of prostacyclin in these patients, the magnitude of the increase in prostacyclin after administration of the synthase inhibitor is substantially less than the maximum capacity of vascular tissue to generate prostacyclin (130)(288)(290) and would not result in levels sufficient to exert systemic effects (132). Nevertheless, endogenous prostacyclin, which has the ability not only to inhibit platelet aggregation in response to all agonists but also to disaggregate previously aggregated platelets (15), may contribute to the efficacy of thromboxane synthase inhibitors by its actions at a local level.

The present study has demonstrated that administration of a thromboxane synthase inhibitor in a dosage schedule representative of those used in clinical trials caused reversible inhibition of thromboxane formation in patients with platelet activation in vivo and enhanced endogenous biosynthesis of thromboxane. Cumulative, complete inhibition of thromboxane biosynthesis did not, however,



occur and platelet activation persisted during chronic drug administration. The results of this study indicate that, as suggested by the investigations in healthy individuals, realization of the therapeutic potential of thromboxane synthase inhibitors is likely to depend on prolongation of drug action and combination with antagonists of the endoperoxide/thromboxane  $A_2$  receptor. In addition, they highlight the importance of achieving complete inhibition of thromboxane formation in order to prevent continuing platelet activation in vivo in such patients.

## II. INHIBITION OF THROMBOXANE BIOSYNTHESIS BY FISH-OIL

### ADMINISTRATION

Epidemiological studies of Greenland Eskimos and mainland Danes have suggested that a diet rich in marine lipids may be associated with a reduction in the incidence of occlusive vascular disease (278)(454). More recently, studies based on dietary history indicate that even a small intake of fish may have reduced the incidence of coronary vascular disease in both European and North American study populations (455)(456). Fish-oil contains the N-3 polyunsaturated fatty acid, eicosapentaenoic acid. Whereas thromboxane  $A_2$  is the predominant product of arachidonic acid formed in platelets, the corresponding product when eicosapentaenoic acid is utilized as substrate is thromboxane  $A_3$  (see Figure 1.11), which is virtually biologically inert (8)(311). The mechanism of the reduced risk of atherosclerotic cardiovascular disease in populations consuming a diet rich in marine oils has therefore been postulated to reflect competition between eicosapentaenoic and arachidonic acid as potential substrates for cyclooxygenase with a resultant fall in the rate of thromboxane  $A_2$  generation. Although substrate competition might also be expected to reduce the

amount of prostacyclin ( $\text{PGI}_2$ ) synthesized, in fact studies in healthy volunteers suggest that endogenous prostacyclin production is preserved during chronic fish-oil administration (312)(457). In addition,  $\text{PGI}_3$ , which is formed in small amounts during dietary supplementation with eicosapentaenoic acid (457)(312), is, like prostacyclin, a potent vasodilator and platelet inhibitor (311).

Studies of fish-oil administration in normal volunteers have reported variable effects on platelet function in these individuals (315-318)(321-323)(447). However, this is likely to reflect an inadequate intake of fish-oil in these studies as the majority have shown a much lower ratio of eicosapentaenoate to arachidonate in the platelet membrane lipids (315-318)(321)(322)(447) than that reported in Eskimos (279). The present study was designed to investigate the mechanisms underlying the effects of fish-oil on platelet and vascular function in patients with platelet activation in vivo and increased biosynthesis of thromboxane. The study had two principal aims. Firstly, to simulate an Eskimo diet in these

patients and relate the biosynthesis of thromboxane and prostacyclin to indices of platelet function in vivo; and secondly, in additional studies in two of the patients, to determine whether inhibition of thromboxane formation could be sustained by lower, more palatable doses of fish-oil.

#### STUDY DESIGN:

##### Patient selection:

Six men (aged 51-69 years) with advanced atherosclerotic peripheral vascular disease participated in the study. The patients were selected from amongst those attending the vascular clinic of the Veterans Administration Hospital, Nashville, Tennessee. All the patients had angiographically-confirmed peripheral arterial disease and met predetermined criteria consistent with the presence of platelet activation in vivo: plasma B-thromboglobulin > 20 ng/ml, with a normal platelet factor 4 level (below 18 ng/ml), and an elevated CPA ratio. Five of the six patients had taken part in the preceding study investigating the effects of the

thromboxane synthase inhibitor, CGS 13080. Two of the patients had diabetes mellitus and 4 were chronic cigarette smokers. Four had electrocardiographic evidence of chronic myocardial ischaemia but none was on anti-anginal medication. All the patients abstained from aspirin or other non-steroidal anti-inflammatory drugs prior to and during the study.

Protocol:

All the participants were given 50ml (10g of eicosapentaenoate) of Max-EPA fish oil (kindly provided by Dr Desmond Davies, R.P. Scherer Company. Troy, Michigan) daily for four weeks as a supplement to their usual diet. To address further the relationship between the dose of fish-oil and the effect on thromboxane biosynthesis, two of the patients were given a lower dose (1g daily) for 6 months after the study with the higher dose (10g per day) had been completed. Compliance was checked by documenting changes in the erythrocyte lipid composition of all the patients.

Urine was collected in 24-hour aliquots prior to the study and at weekly intervals during supplementation for measurement of 2,3-dinor thromboxane B<sub>2</sub> (Tx-M) and

2,3-dinor-6-keto-PGF<sub>1α</sub> (PGI-M) and to determine whether the corresponding metabolites of thromboxane A<sub>3</sub> (2,3-dinor-17-ene-thromboxane B<sub>2</sub>) and prostaglandin I<sub>3</sub> (2,3-dinor-6-keto-17-ene-PGF<sub>1α</sub>) were excreted. Blood was drawn before the study and weekly during the study for measurement of serum thromboxane B<sub>2</sub>, B-thromboglobulin, platelet factor 4, the circulating platelet aggregate ratio, platelet aggregation studies, serum α-tocopherol and the full blood count. In addition, incorporation of eicosapentaenoic acid into red cell phospholipid fractions and routine clotting studies (prothrombin time, kaolin cephalin clotting time and thrombin time) and serum chemistry were determined prior to and during the last week of supplementation. The bleeding time was measured before and at weekly intervals during supplementation and three months after Max-EPA had been discontinued. The patients were also evaluated clinically by direct questioning regarding the severity of their symptoms and by physical examination.

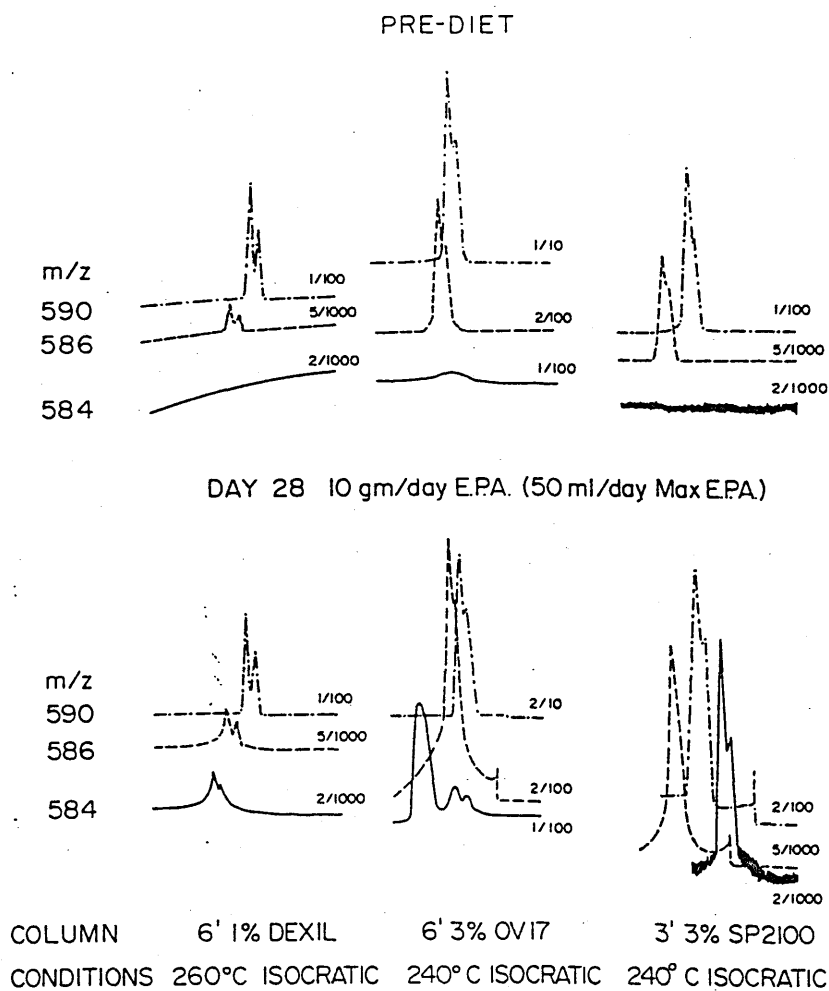
#### Analytical Methods:

The presence of platelet activation in vivo was assessed by measurement of plasma B-thromboglobulin and platelet factor 4 by radioimmunoassay and of the CPA

ration by a modification of the method of Wu and Hoak (350). In addition, Tx-M and PGI-M were determined by a stable isotope dilution assay employing gas chromatography/mass spectrometry as described in chapter 2. The dinor metabolites of thromboxane  $B_3$  ( $TxB_3$ -M) and prostaglandin  $I_3$  ( $PGI_3$ -M) were quantified simultaneously by selected ion monitoring at 2 mass units lower ( $m/z$  584) because of the extra double bond (457). Representative selected ion monitoring traces before and after fish-oil supplementation are shown in Figure 5.11). Platelet aggregation in PRP was tested in response to arachidonic acid (0.66mM) and serum thromboxane  $B_2$  was determined by radioimmunoassay. The bleeding time was measured by the template method.

Analysis of erythrocyte phospholipids and serum  $\alpha$ -tocopherol were kindly performed by Patricia Price of the Department of Pharmacology, Vanderbilt University. Serum  $\alpha$ -tocopherol was measured by high-performance liquid chromatography (458). Erythrocyte phospholipids were extracted according to the Folch procedure (458), purified by thin-layer chromatography by a modification of Skipski's method (460)(461) and analysed with a gas chromatograph equipped with an integrator (Varian 2100, Varian Corporation, Palo Alto, California) on a 2-metre column of internal diameter 2mm packed with 10% SP 2340 on

FIGURE 5.11 SELECTED ION MONITORING TRACES OF URINARY EICOSANOID METABOLITES BEFORE/AFTER FISH-OIL ADMINISTRATION.





Supelcoport (Supelco, Bellefonte, Pennsylvania).

## RESULTS:

### Evidence of Platelet Activation in vivo:

The patients all had evidence of platelet activation in vivo. B-thromboglobulin was elevated at  $60 \pm 17$  ng/ml (normal:  $13 \pm 3$  ng/ml) with a normal platelet factor 4 level ( $8 \pm 1$  ng/ml). The CPA ratio was also increased at  $1.30 \pm 0.08$  (normal:  $1.0 \pm 0.02$ ) and the bleeding time was shortened at  $3.3 \pm 0.2$  minutes (normal:  $5.0 \pm 0.4$  minutes). Urinary excretion of both Tx-M and PGI-M was enhanced at  $1406 \pm 245$  and  $364 \pm 71$  pg/mg creatinine respectively (normal:  $223 \pm 22$  pg/mg creatinine for Tx-M and  $197 \pm 21$  pg/mg creatinine for PGI-M). The normal values are derived from a population of healthy individuals of the same age (see Appendix I).

### Effects on Thromboxane Synthesis in vivo and ex vivo:

There was a rapid and marked fall in Tx-M excretion during the first week of fish-oil ingestion from  $1406 \pm 245$  to  $840 \pm 101$  pg/mg creatinine ( $p < 0.05$ ). Tx-M

continued to fall during the study by a maximum of  $58 \pm 8$  % to  $588 \pm 142$  pg/mg creatinine ( $p < 0.01$ ) by the end of the fourth week (Figure 5.12). No detectable excretion of the dinor metabolite of thromboxane  $B_3$ ,  $TxB_3-M$ , occurred before or during the first week of the study period, but a minor accumulation was found by the end of the fourth week ( $49 \pm 7$  pg/mg creatinine;  $p < 0.001$ ) (Figure 5.11). There was also a significant reduction ( $p < 0.05$ ) in serum thromboxane generation during supplementation from  $429 \pm 38$  to  $242 \pm 91$  ng/ml, a fall of  $44 \pm 10$  %. This accompanied a slight reduction in the platelet count in all the patients during the study from  $268 \pm 15$  to  $240 \pm 21 \times 10^9/l$  (Table 5.6), which failed to reach statistical significance.

The effects on Tx-M excretion of the lower dose of fish-oil in the two patients who were given 1g daily for six months are shown in Figure 5.13. There was a gradual return in Tx-M excretion, which had fallen during the higher dose of fish-oil, back to the elevated pretreatment values. This was accompanied by a decline in the eicosapentaenoate content of their erythrocyte phosphatidylcholines from 8.9% immediately after the high dose of fish-oil to 3.3% and 1.4% at three and six months respectively of supplementation (pretreatment  $< 0.5\%$ ).

FIGURE 5.12 URINARY EXCRETION OF METABOLITES OF THROMBOXANES AND PROSTACYCLINS BEFORE AND DURING FISH-OIL INGESTION (10g daily) IN PATIENTS WITH ATHEROSCLEROSIS.

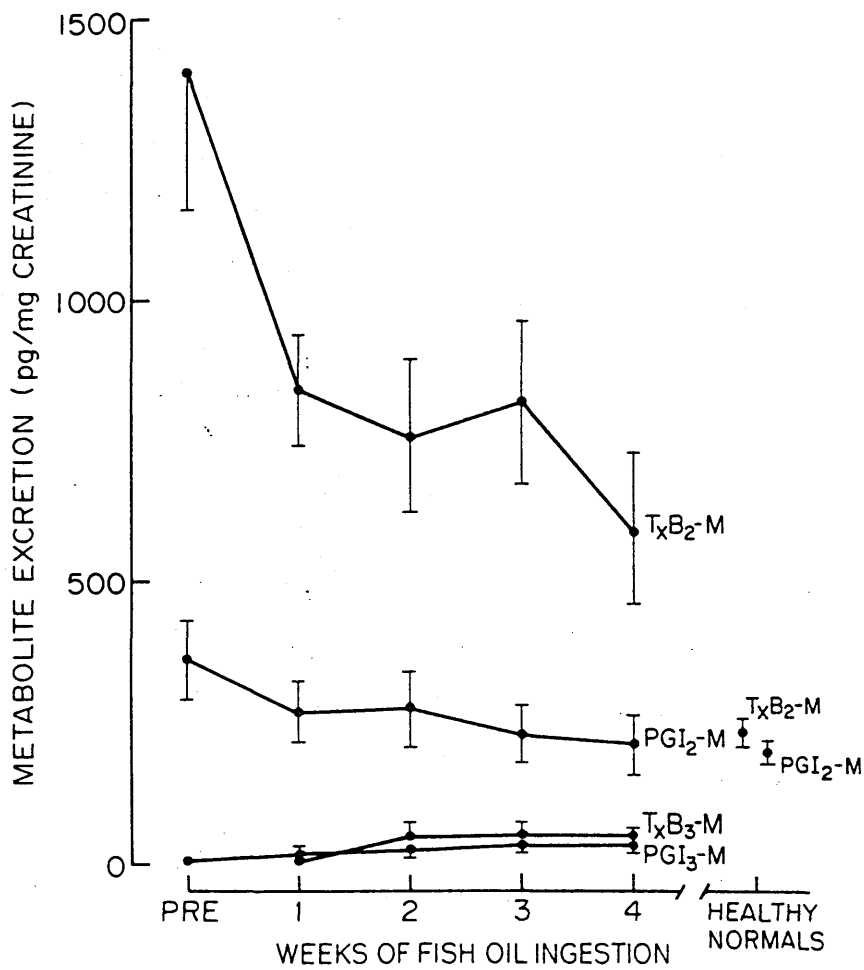


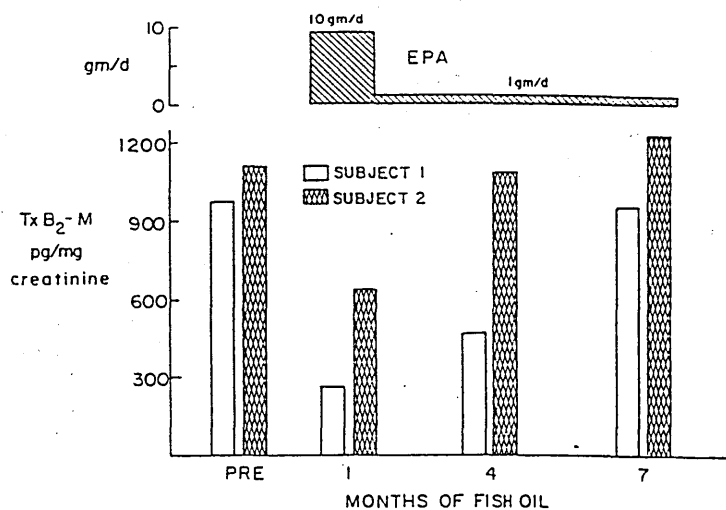
TABLE 5.6 EFFECT OF FISH-OIL ON INDICES OF PLATELET FUNCTION IN PATIENTS WITH ATHEROSCLEROSIS AND PLATELET ACTIVATION IN VIVO.

	CPA Ratio	Platelet Count ( $\times 10^9/l$ )	Platelet aggregation in response to 0.66mM arachidonic acid (LT <sub>50</sub> , minutes)
Before treatment:	1.28 $\pm$ 0.08	268 $\pm$ 15	0.58 $\pm$ 0.04
Treatment:			
Week 1	1.05 $\pm$ 0.02*	280 $\pm$ 19	0.47 $\pm$ 0.05
Week 2	1.26 $\pm$ 0.09	252 $\pm$ 22	0.56 $\pm$ 0.04
Week 3	1.05 $\pm$ 0.03	240 $\pm$ 21*	0.46 $\pm$ 0.03
Week 4	1.16 $\pm$ 0.07	254 $\pm$ 34	0.56 $\pm$ 0.05
After treatment:	1.27 $\pm$ 0.08	291 $\pm$ 14	0.51 $\pm$ 0.01

\*  $p < 0.05$

Values are mean  $\pm$  SEM of determinations in 6 male patients.

FIGURE 5.13 Tx-M EXCRETION AFTER ADMINISTRATION OF A LOWER (1g) DOSE OF FISH OIL TO TWO PATIENTS WITH ATHEROSCLEROSIS.



#### Effects on Prostacyclin Biosynthesis:

PGI-M excretion fell within the first week of supplementation from 364  $\pm$  71 to 269  $\pm$  54 pg/mg creatinine (Figure 5.12). This initial decline continued throughout the study period to reach a significant ( $p < 0.05$ ) mean decrement of 42% at 212  $\pm$  57 pg/mg creatinine at the end of the fourth week. A small quantity of PGI<sub>3</sub>-M was detected in all the patients before fish-oil ingestion (7  $\pm$  1 pg/mg creatinine) and increased significantly ( $p < 0.01$ ) during supplementation to a peak of 30  $\pm$  9 pg/mg creatinine by the fourth week.

#### Effects on Platelet Function:

The bleeding time was significantly prolonged ( $p < 0.01$ ) in all the patients by the end of the first week of treatment from 3.3  $\pm$  0.2 to 4.6  $\pm$  0.4 minutes and remained so for the duration of supplementation (Figure 5.14). Three months after supplementation the bleeding time had returned to pretreatment values (3.4  $\pm$  0.6 minutes). Plasma concentrations of B-thromboglobulin also fell during the study from 60  $\pm$  17 to 35  $\pm$  35 ng/ml at the end of the fourth week. The effect of fish-oil

FIGURE 5.14 EFFECT OF FISH-OIL ON THE BLEEDING TIME IN PATIENTS WITH ATHEROSCLEROSIS.

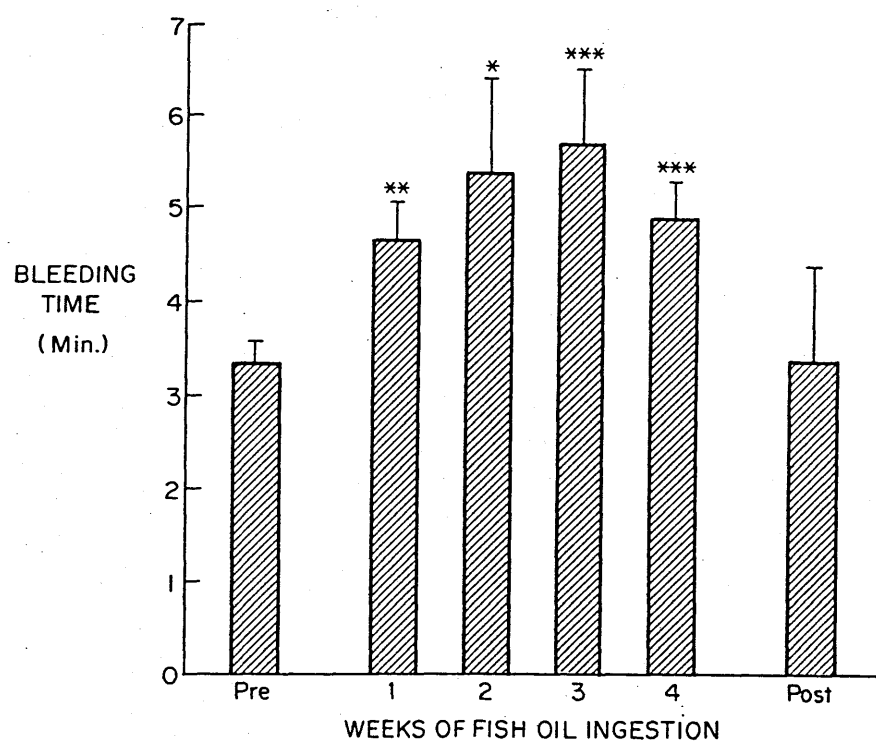
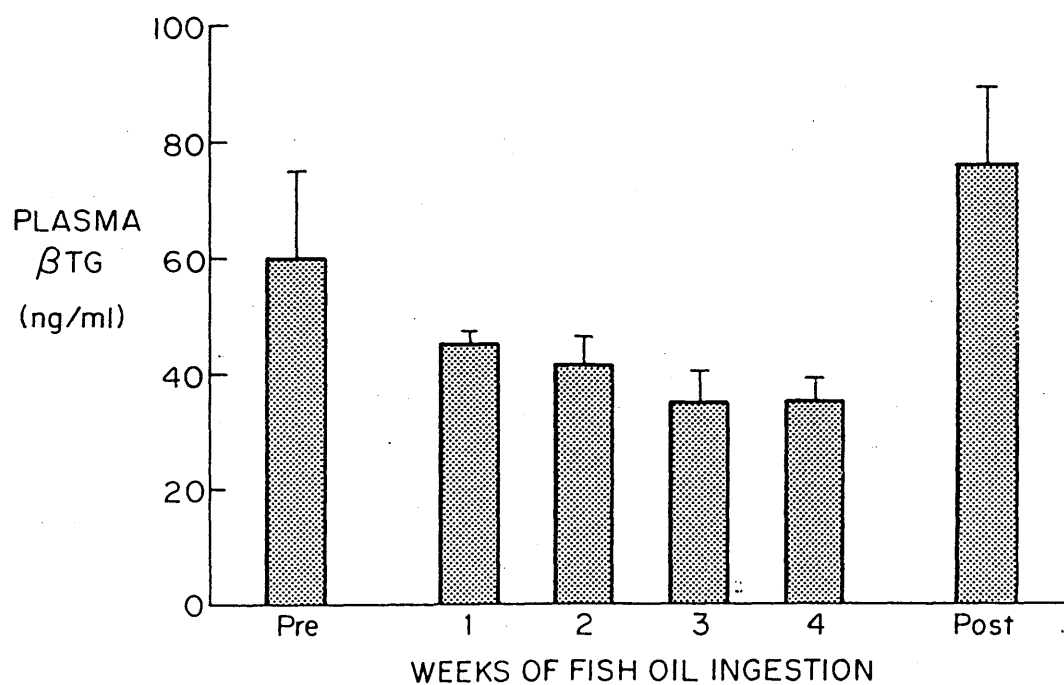


FIGURE 5.15 EFFECT OF FISH-OIL ON PLASMA B-THROMBOGLOBULIN IN PATIENTS WITH ATHEROSCLEROSIS.



ingestion was shown to be significant by analysis of variance ( $p < 0.001$ ), but became significant by pairwise comparisons ( $p < 0.05$ ) only at the third week (Figure 5.15). There was no change in plasma levels of platelet factor 4 or in arachidonate-induced platelet aggregation during the study (Table 5.6). The CPA ratio fell from  $1.3 \pm 0.08$  to  $1.05 \pm 0.02$  at the end of the first week of supplementation but the values fluctuated during treatment and were significant ( $p < 0.05$ ) only after the first and third weeks of the study (Table 5.6).

#### Effects on Erythrocyte Fatty Acids:

There was extensive incorporation of N-3 fatty acids into erythrocyte phospholipids during dietary supplementation with eicosapentaenoate (Table 5.7). By the end of the fourth week, the ratio of eicosapentaenoic to arachidonic acid had risen from 0.08 to 1.24 in erythrocyte phosphatidylcholines and from  $<0.02$  to 0.23 and 0.06 in phosphatidyl ethanolamines and phosphatidyl (serines plus inositols) respectively. In common with the effects on thromboxane and prostacyclin biosynthesis and platelet function, the changes largely occurred during the first week of treatment. The baseline values were similar to those reported in other studies (280)(281)(323)(457),

but showed more extensive incorporation of eicosapentaenoate consistent with the higher dose used in this study.

TABLE 5.7 INCORPORATION OF EICOSA PENTAENOIC ACID (EPA) AND ARACHIDONIC ACID (AA) INTO ERYTHROCYTE PHOSPHOLIPIDS IN PATIENTS WITH ATHEROSCLEROSIS AND PLATELET ACTIVATION IN VIVO.

Phospholipid	Before Treatment		After Treatment		
	AA	EPA	AA	EPA	EPA/AA Ratio
Phosphatidylcholines	6.5±0.8	<0.5	5.0±0.4	6.2±1.0	1.24
Phosphatidylethanolamines	24.3±0.8	<0.5	19.5±0.3	4.5±0.2	0.23
Phosphatidyl(serines plus inositols)	27.6±0.5	<0.5	25.7±0.9	1.5±0.2	0.06

Values are expressed as percentages of total fatty acids

(mean ± SEM) in 6 male patients.

### Clinical Evaluation:

All the patients tolerated the fish-oil without adverse effects. There was no objective evidence of symptomatic improvement in any of the subjects during the study nor were there any alterations in pedal blood flow, including skin colour, temperature, capillary refill time and time or extent of postural changes in skin colour using Buerger's postural test. There was no significant reduction in serum  $\alpha$ -tocopherol (8.5 +/- 1.5 vs 10.9 +/- 2.2 ug/ml, before vs after treatment) and no changes in



the routine serum chemistry screens or clotting studies were noted.

### Discussion

This study has demonstrated that fish-oil supplementation, using a diet which simulated the fish content of an Eskimo diet, significantly reduced the elevated excretion of Tx-M in patients with severe atherosclerosis and platelet activation in vivo. However, endogenous biosynthesis of thromboxane was not completely inhibited and remained at levels significantly above those found in healthy individuals. This was consistent with the moderate effects observed on other indices of platelet function in vivo and was similar to the degree of inhibition achieved with a thromboxane synthase inhibitor in the same model. Moreover, there was a concomitant return in thromboxane biosynthesis to pretreatment values as the eicosapentaenoic acid content of erythrocyte phospholipids declined when patients were changed to a

lower, more palatable dose of fish-oil. The alterations in endogenous thromboxane formation have therefore been shown to relate directly to the red cell membrane incorporation of eicosapentaenoate which in turn is determined by the dose and duration of fish-oil administration.

There was also a reduction in prostacyclin biosynthesis during chronic administration of fish-oil to these patients. This is in contrast to healthy individuals in whom endogenous production of prostacyclin is maintained (315)(457). The corresponding trienoic compound,  $\text{PGI}_3$ , was formed to a similar extent in the patients with atherosclerosis to the levels seen in normal subjects (457). These observations, and the concomitant fall in B-thromboglobulin and prolongation of the bleeding time, are consistent with a reduction of platelet activation in vivo in these patients in response to fish-oil. The reduction in prostacyclin biosynthesis was accompanied by a much greater depression of endogenous thromboxane formation. Thus, inhibition of platelet activation leading to reduced thromboxane generation appears to suppress the stimulus to enhanced prostacyclin biosynthesis in these patients. This provides further evidence that the increased production of prostacyclin seen in this and other human syndromes of platelet activation in vivo (see chapter 4 and reference 422), reflects an enhanced

frequency or intensity of platelet-vessel wall interactions (180).

There is little information available relating the dose of fish-oil to its effects on platelets. While previous studies in healthy volunteers have reported only minor or insignificant inhibition of platelet function, most of these have employed too low a dietary content of eicosapentaenoic acid and have therefore attained a ratio of eicosapentaenoate to arachidonate in membrane lipids of 0.3 or less (315-318)(321)(322)(447). In this study, as in the membrane lipids of Eskimos (279), this ratio was much higher (greater than 1.0). The results of the present study also showed that a reduced dose of fish-oil, 1g daily, was insufficient to maintain the platelet-inhibitory effects achieved with the higher dose, even when treatment was continued for six months. Similarly, in a study of survivors from myocardial infarction, Saynor et al found that the bleeding time was significantly prolonged by 3.6g, but not 1.8g, of eicosapentaenote daily for a year (448). More recently others have also reported dose-related effects on platelet aggregation in volunteers who ingested cod-liver oil (449).

These results suggest that higher doses of fish-oil, at

least equal to those employed in the present investigation, will be required to significantly influence platelet function in patients with platelet activation in vivo and enhanced endogenous biosynthesis of thromboxane. The considerable depression of thromboxane formation which accompanied the changes in other indices of platelet activation using this regimen, strongly suggests that the beneficial effects of fish-oil are primarily the result of alterations in endogenous eicosanoid biosynthesis. Nevertheless, other properties of fish-oil, such as its effects on membrane fluidity (326), whole blood viscosity (462), plasma lipids (325) and neutrophil function (463), may confer additional advantages on this therapeutic approach. In this regard, the results of two recent studies, in which administration of fish-oil was found to inhibit the development of atherosclerosis in Rhesus monkeys fed an atherogenic diet (464) and in a hyperlipidaemic swine model (465), are particularly interesting.

## CONCLUSION

The studies described in this chapter have investigated the effects of two distinct approaches to the inhibition of thromboxane formation in a human model of platelet activation in vivo in which thromboxane biosynthesis was markedly enhanced. The model chosen, patients with advanced atherosclerotic disease, was selected both because it was one in which there was clear evidence of thromboxane-dependent platelet activation and also because it represents an extremely common clinical problem in which therapeutic intervention has generally yielded disappointing results.

The principal conclusion which can be drawn from these studies is that although both of the regimens employed were successful in considerably reducing endogenous thromboxane formation, platelet activation in vivo persisted. In addition, while the numbers studied were small and the methods used to assess efficacy were limited, the presence of continuing platelet activation was accompanied by a failure to demonstrate significant clinical benefit. Increasing evidence from biochemical, pathological and pharmacological studies implicates

platelets in the pathogenesis of vascular occlusive disease and there is growing interest in particular in the role of platelet-derived growth factors released during platelet activation. The studies described in this chapter indicate that in order to suppress platelet activation in vivo, therapeutic regimens must be directed towards achieving virtually complete inhibition of thromboxane biosynthesis throughout the dosing interval. Large-scale studies will, however, be necessary to establish the clinical value of this approach.

I acknowledge the help of Ms Johniene Doran with some of the platelet function tests carried out in these studies.

## Chapter 6

### FINAL DISCUSSION AND CONCLUSION

The potent biological properties of thromboxane  $A_2$  and its putative role in vascular occlusion provide the rationale for therapeutic regimens designed to reduce thromboxane synthesis in man. This can be readily achieved in vitro and in vivo by aspirin. However, concern about the functional significance of concomitant inhibition by aspirin of the platelet-inhibitory eicosanoid, prostacyclin, has stimulated interest in the development of selective approaches to reducing thromboxane production. The studies described in this work have investigated the human pharmacology of the different methods of achieving this objective.

The biochemical and functional effects of a new thromboxane synthase inhibitor, 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid (UK 38,485), were evaluated in healthy volunteers. The thromboxane synthase inhibitor efficiently blocked platelet thromboxane synthesis ex vivo confirming the potency of this group of drugs in reducing platelet capacity for thromboxane generation. In addition, in the higher dose, the synthase inhibitor significantly



increased endogenous prostacyclin synthesis, consistent with diversion of platelet-derived prostaglandin endoperoxides accumulated in the presence of thromboxane synthase inhibition to be utilized by a source of prostacyclin synthase. Despite these findings, the effects of the thromboxane synthase inhibitor on platelet function were relatively minor and transient.

The reasons for this apparent dilemma were explored by comparing the effects of the synthase inhibitor with those of a "low" (120mg) dose of aspirin. The peak reduction in serum thromboxane  $B_2$  in response to aspirin was almost identical to that achieved with the synthase inhibitor. However, the significantly greater platelet-inhibitory activity of this low dose of aspirin, together with in vitro studies of platelet behaviour in response to thromboxane synthase inhibitors, suggest that accumulated prostaglandin endoperoxides substitute for the proaggregatory effects of thromboxane when arachidonate metabolism via thromboxane synthase is blocked. Since even in this low dose aspirin also significantly inhibited prostacyclin formation, and the corresponding decline in thromboxane formation was far from optimal (around 30%), these results indicate that a truly selective, effective single dose of aspirin was not achieved.

Two new and potentially useful approaches, which were selective with respect to their effects on eicosenoid formation, were identified in these studies. The combination of low-dose aspirin with a thromboxane synthase inhibitor appeared to offset the principal disadvantages of either drug administered alone. Thus the fall in prostacyclin biosynthesis seen after low-dose aspirin was no longer evident and there was substantial and prolonged inhibition of platelet function. The second approach employed repeated administration of extremely low doses of aspirin (1mg every 30 minutes) to achieve selective presystemic acetylation of platelet cyclooxygenase. It was postulated that, in such a dose, hepatic extraction of aspirin would be essentially complete and vascular cyclooxygenase would therefore not be exposed to significant concentrations of aspirin. Consistent with this hypothesis, aspirin remained undetectable in peripheral plasma throughout the study, prostacyclin biosynthesis was unaltered and the rapid recovery of serum thromboxane  $B_2$  suggested that megakaryocyte cyclooxygenase was also unaffected.

The possibility that the minor effects of UK 38,485 on platelet function might also result from incomplete inhibition of actual thromboxane synthesis in vivo was investigated by measuring the excretion of Tx-M

(2,3-dinor-thromboxane  $B_2$ ), a major urinary metabolite of thromboxane. Experiments with a range of doses of aspirin alone and in combination with UK 38,485, showed that the relationship between actual thromboxane biosynthesis (Tx-M) and the capacity for thromboxane generation ex vivo (serum thromboxane  $B_2$ ) was not linear. Following a single dose of UK 38,485 Tx-M fell by less than 30% despite a corresponding reduction in serum thromboxane  $B_2$  of over 95%. Thus, inhibition of thromboxane formation in vivo may be far from optimal despite apparently maximal depression of serum thromboxane  $B_2$ . This observation has important implications for platelet-inhibitory therapy in patients with platelet activation in whom it is likely that pharmacological inhibition of serum thromboxane would have to be virtually complete before thromboxane-dependent platelet aggregation is influenced in vivo.

In order to evaluate the likely impact of selective inhibition of thromboxane synthesis in a clinical setting, an appropriate human model of thromboxane-dependent platelet activation in vivo was sought. Evidence of platelet activation and markedly enhanced biosynthesis of thromboxane was found in patients with severe atherosclerosis and, to a lesser degree, in patients with systemic sclerosis and Raynaud's phenomenon. Moreover, an

additional stimulus to platelet activation (the precipitation of Raynaud's phenomenon on exposure to cold) in the patients with systemic sclerosis, resulted in a further increment in thromboxane formation. In each case the increase in thromboxane biosynthesis was accompanied by a significant though smaller increment in endogenous prostacyclin production, consistent with an increased frequency or intensity of platelet-vascular interactions. Other indices of platelet activation in vivo (elevated plasma B-thromboglobulin, the presence of circulating platelet aggregates and a shortening of the bleeding time) were also present. These studies confirmed the value of Tx-M as a sensitive, non-invasive index of platelet activation in vivo, both in the setting of chronic disease and in response to acute changes.

The effects of selective inhibition of thromboxane formation were then investigated in a group of patients with severe atherosclerosis, chosen to fulfil preset criteria for the presence of platelet activation in vivo and with markedly enhanced endogenous biosynthesis of thromboxane. Two different approaches were evaluated: specific blockade of thromboxane synthase by imidazo-(1,5-2)-pyridine-5-hexanoic acid (CGS 13080), and an indirect approach in which dietary supplementation with fish-oil was used to inhibit platelet activation and

reduce thromboxane formation. The decision to use a thromboxane synthase inhibitor, despite the apparently inferior platelet-inhibitory properties of these compounds, was based on the recognition that these are the only drugs known to increase endogenous prostacyclin production. Dietary supplementation with fish-oil was similarly hoped to enhance the formation of endogenous vasodilator and platelet-inhibitory eicosanoids. Previous studies in healthy individuals had shown that synthesis of prostaglandin  $I_3$ , which has similar biological activity to prostacyclin, was increased by fish-oil.

Administration of the thromboxane synthase inhibitor, in a dose representative of those used in clinical trials, caused reversible inhibition of platelet thromboxane formation in these patients and also significantly increased endogenous prostacyclin production more than two-fold. Nevertheless, cumulative complete inhibition of thromboxane biosynthesis was not achieved and platelet activation persisted during chronic drug administration. This was found to be due in part to the pharmacokinetics of these drugs. Significant recovery of platelet thromboxane formation was shown to occur within the 6-hour dosing interval, reflecting the short pharmacological half-lives which are common to all the thromboxane synthase inhibitors under investigation in man. However,

the superior anti-platelet activity of aspirin in the same patients, and the failure of the thromboxane synthase inhibitor to influence platelet function at the time of maximal drug action, suggested that the substitution by accumulated endoperoxides for the proaggregatory effects of thromboxane  $A_2$  also contributed to continuing platelet activation in these patients. It therefore seems likely that realization of the therapeutic potential of thromboxane synthase inhibitors will depend both on prolongation of drug action and on their combination with antagonists of the endoperoxide/thromboxane  $A_2$  receptor.

Dietary supplementation with 10g daily of eicosapentaenoate for one month also significantly reduced thromboxane biosynthesis in patients with severe atherosclerosis and platelet activation in vivo. The decline in thromboxane  $A_2$  production coincided with the formation of inactive thromboxane  $A_3$  and increased eicosapentaenoate incorporation into erythrocyte phospholipids. Prostaglandin  $I_3$  biosynthesis was also significantly increased. Inhibition of thromboxane generation was not complete, however, and platelet activation in vivo although significantly reduced was still apparent. A lower dose of eicosapentaenoate (1g daily) was insufficient to maintain the changes in

thromboxane synthesis. Although chronic administration of the high dose will be required to modify eicosenoid formation and platelet function, other biological properties of fish-oil, particularly its potential ability to prevent the development of atherosclerosis, make this approach worthy of further investigation.

In conclusion, these studies have shown that in several syndromes of platelet activation in vivo, thromboxane biosynthesis is markedly increased. Such syndromes provide an appropriate model to investigate the effects of regimens to inhibit thromboxane formation in man. It is apparent that in order to suppress platelet activation in patients with these conditions, therapeutic regimens must achieve virtually complete inhibition of thromboxane biosynthesis throughout the dosing interval. The observation that prostacyclin synthesis is also enhanced in this setting supports the development of selective approaches to inhibition of thromboxane generation which spare, or even increase, prostacyclin production. These studies suggest that efforts should be directed towards the synthesis of new, long-acting thromboxane synthase inhibitors to be used in combination with thromboxane receptor antagonists. In addition, the potential benefits of extremely low dose 'slow-release' preparations of

aspirin and of long-term administration of pharmacological doses of eicosapentenoate need to be investigated in controlled clinical trials in vasoocclusive disease.



## APPENDIX I

### Prostaglandin Metabolites

:normal values

Serum Thromboxane B<sub>2</sub>:

Normal values

Effect of age

Effect of sex

Effect of platelet count

Urinary Tx-M and Thromboxane B<sub>2</sub>:

Normal values

Effect of sex

Intraindividual variation

Effect of age

Urinary PGI-M:

Normal values

Intraindividual variation

Effect of age

TABLE AI.1 SERUM THROMBOXANE B<sub>2</sub> : NORMAL VALUES (ng/ml)

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	437	384	449	462	425	388	424
TL	314	166	137	119	209	236	197
BS	550	445	455	600	493	509	509
MM	382	470	463	474	389	419	433
WB	779	457	456	424	646	534	549
MA	275	272	339	253	348	230	286
DM	393	400	405	385	347	455	398
NK	225	246	208	233	222	199	222
RR	468	361	385	497	402	375	415
RP	358	299	348	359	389	347	350
PL	341	263	314	298	314	356	314
MV	324	293	260	270	341	299	298

Normal values of serum thromboxane B<sub>2</sub> in 12 healthy male subjects on 6 separate samples.

FIGURE AI.1 RELATIONSHIP BETWEEN SERUM THROMBOXANE  $B_2$  GENERATION EX VIVO AND AGE IN 30 HEALTHY SUBJECTS.

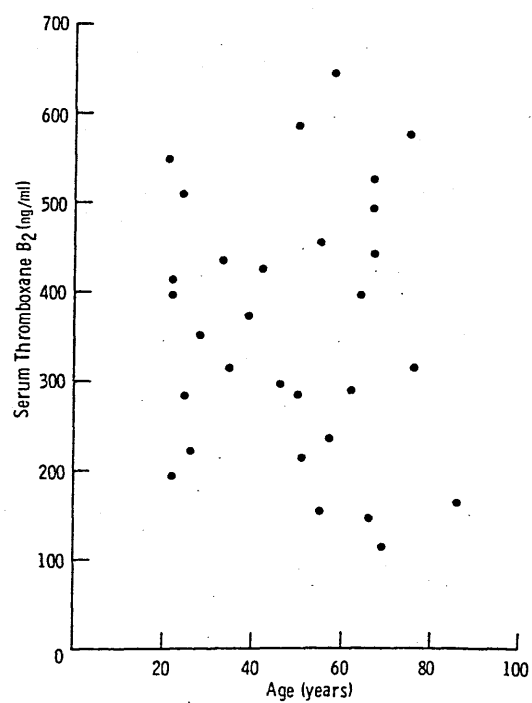


FIGURE AI.2 RELATIONSHIP BETWEEN SERUM THROMBOXANE  $B_2$  AND SEX.

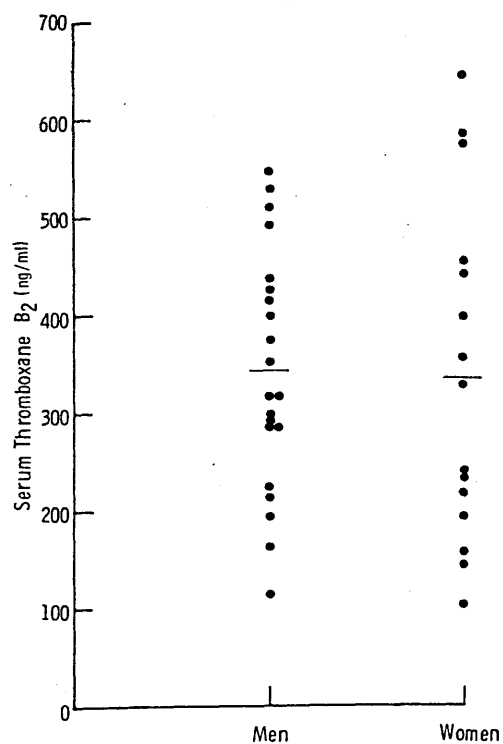


FIGURE AI.3 RELATIONSHIP BETWEEN SERUM THROMBOXANE B<sub>2</sub> GENERATION EX VIVO AND PLATELET COUNT IN 30 HEALTHY SUBJECTS.

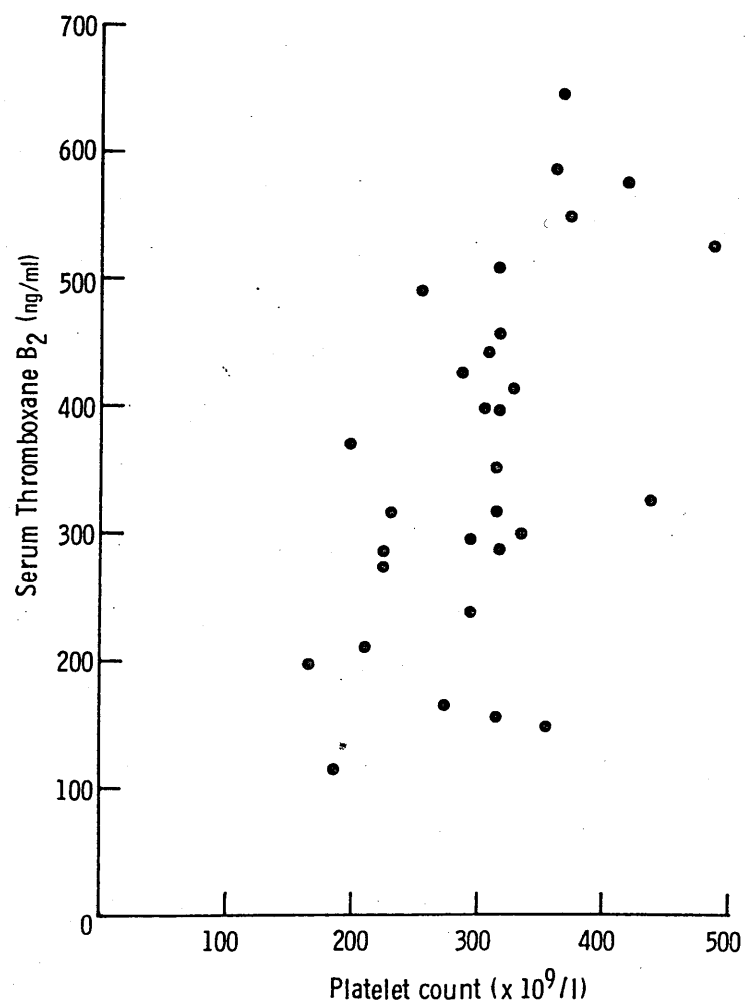


FIGURE AI.4 URINARY EXCRETION OF Tx-M AND THROMBOXANE B<sub>2</sub> : NORMAL VALUES IN HEALTHY SUBJECTS.

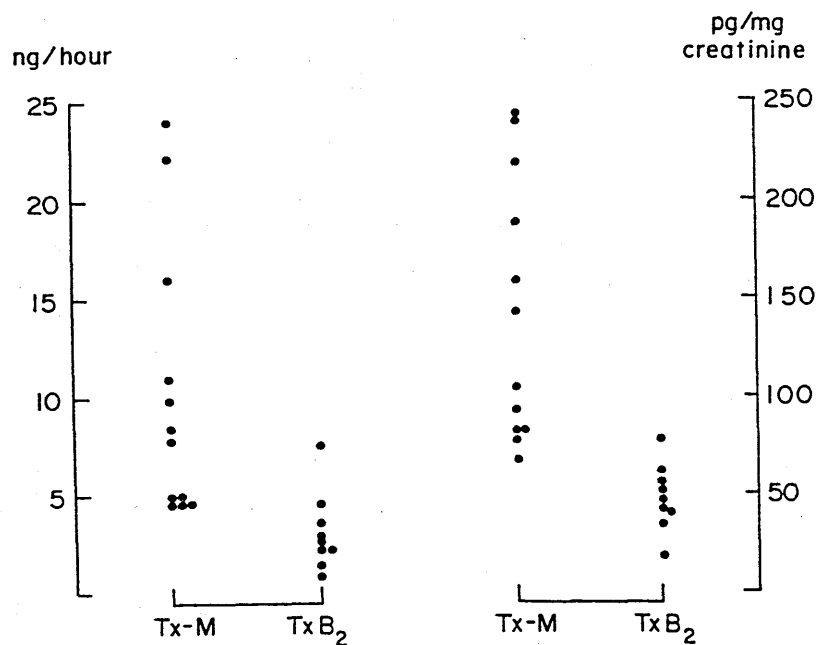
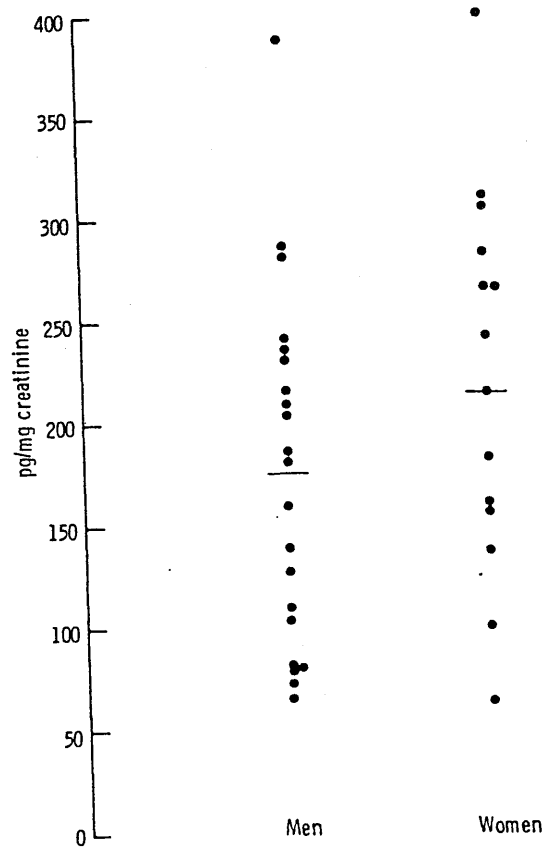


FIGURE AI.5 URINARY EXCRETION OF Tx-M : EFFECT OF SEX.



Subject	Time 1	Time 2	Time 3
BS	62	80	168
TL	84	95	106
WB	61	73	74
CR	152	205	222
DM	58	58	84
PL	130	141	166

2,3- Dinor-Thromboxane B<sub>2</sub>  
(pg/mg creatinine)

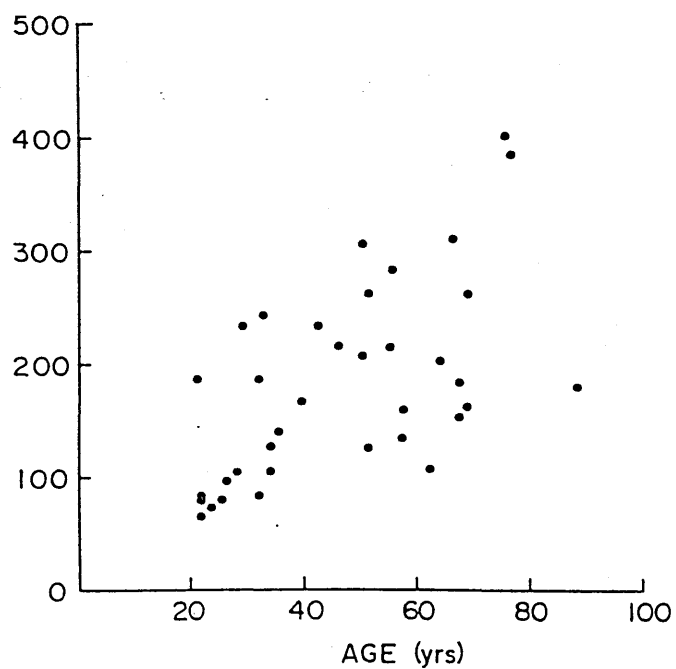


FIGURE AI.8 URINARY EXCRETION OF PGI-M: NORMAL VALUES IN HEALTHY SUBJECTS.

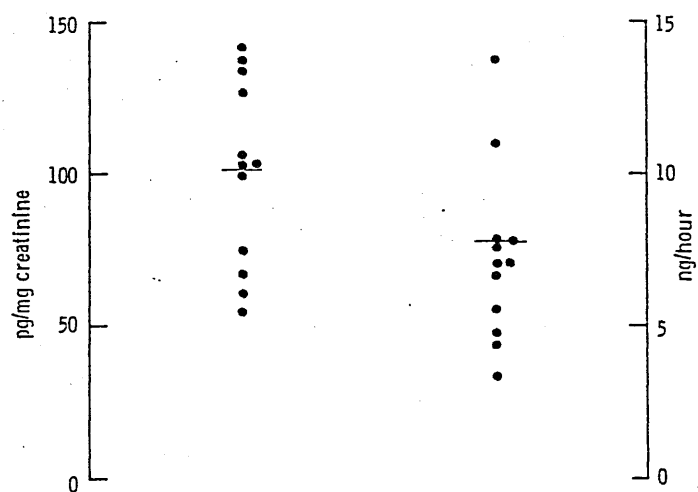


FIGURE AI.9 URINARY EXCRETION OF PGI-M: INTRAINDIVIDUAL VARIATION.

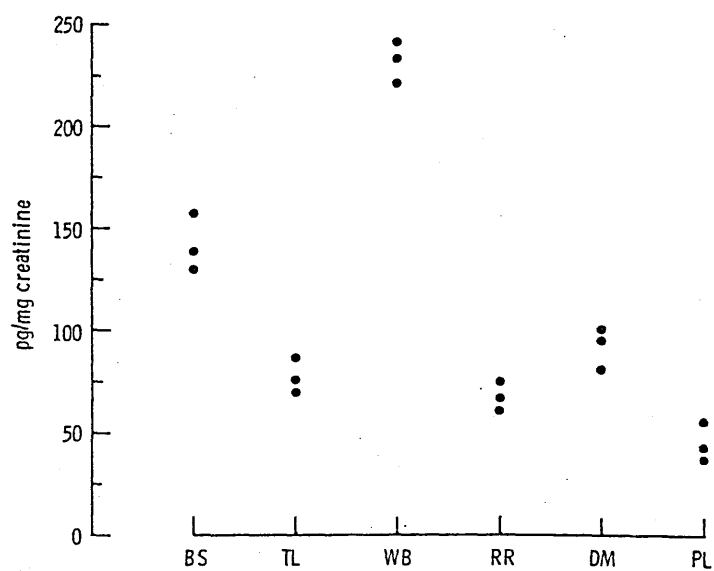
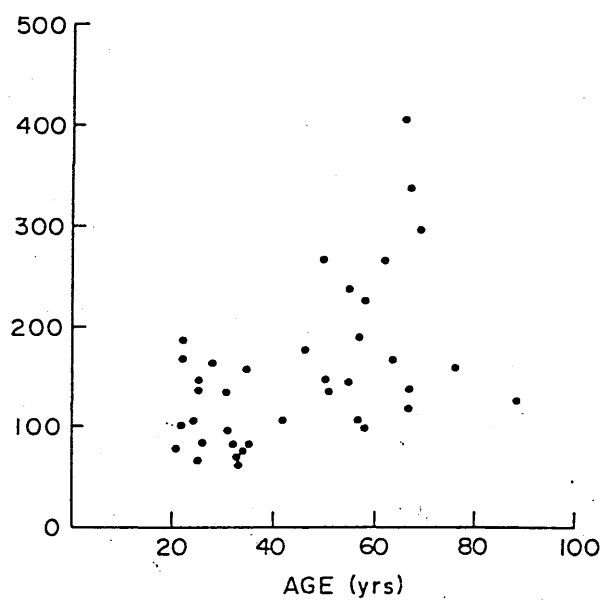




FIGURE AI.10 EFFECT OF AGE ON PGI-M EXCRETION.

2,3-Dinor-6-Keto-PGF<sub>1α</sub>  
(pg/mg creatinine)



## APPENDIX II

### Platelet Function

#### :normal values

Bleeding Time

Platelet Aggregation:

Arachidonic acid

Collagen

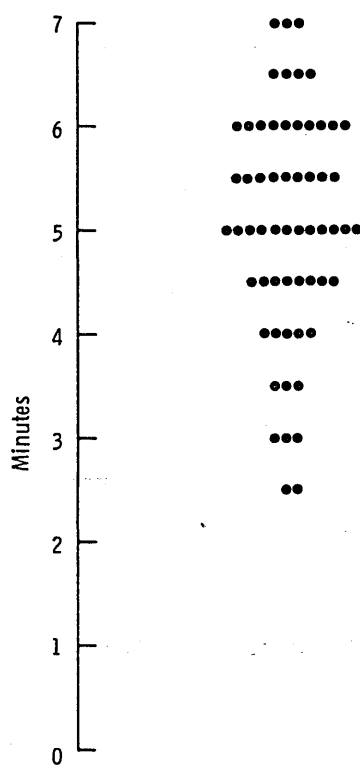
Adrenaline

Whole blood platelet aggregation

Platelet Granule Proteins

Circulating Platelet Aggregates

FIGURE AII.1 TEMPLATE BLEEDING TIME IN 12 HEALTHY MALE SUBJECTS.  
(5 separate estimations on each subject)



**TABLE AII.1 PLATELET AGGREGATION IN PLATELET-RICH PLASMA (PRP)  
IN RESPONSE TO ARACHIDONIC ACID (1.33mM) :  
NORMAL VALUES OF  $LT_{50}$ \* (mins).**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	0.4	0.52	0.56	0.76	0.54	0.64	0.57
TL	1.12	1.04	1.06	0.7	0.72	0.74	0.9
BS	0.62	0.56	0.7	0.48	0.6	0.74	0.61
MM	0.52	0.66	0.76	0.64	0.76	0.73	0.68
WB	0.7	0.84	0.78	0.64	0.5	0.74	0.7
MA	0.64	0.7	0.66	0.6	0.64	0.80	0.67
DM	0.58	0.6	0.54	0.44	0.44	0.6	0.53
NK	0.6	0.52	0.5	0.48	0.82	0.48	0.52
RR	0.56	0.7	0.6	0.62	0.6	0.56	0.61
RP	0.56	0.64	0.6	0.6	0.54	0.62	0.59
PL	0.56	0.6	0.48	0.64	0.78	0.76	0.64
MV	0.68	0.72	0.64	0.74	0.7	0.7	0.7

Normal values for  $LT_{50}$  in 12 healthy male subjects on 6 separate samples.

\*  $LT_{50}$  : Lag time to achieving 50% of maximal aggregation.

NA : no aggregation.

TABLE AII.2 PLATELET AGGREGATION IN PRP IN RESPONSE TO ARACHIDONIC ACID (0.66mM) : NORMAL VALUES OF  $LT_{50}^*$  (mins).

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	0.52	0.58	0.6	0.76	0.6	0.76	0.64
TL	1.36	2.16	1.48	0.76	1.0	1.06	1.3
BS	3.7	4.16	3.04	2.56	3.96	4.72	3.7
MM	0.6	0.7	0.68	0.66	0.84	0.96	0.74
WB	1.8	1.1	0.74	0.64	0.66	1.06	1.0
MA	1.44	1.2	1.45	1.24	1.68	1.68	1.45
DM	1.0	0.8	1.04	0.8	0.72	0.8	0.86
NK	0.86	0.72	0.72	0.72	0.72	0.6	0.72
RR	0.88	0.7	0.62	0.76	0.64	0.76	0.73
RP	0.64	0.58	0.54	0.6	0.58	0.68	0.6
PL	1.0	0.7	0.54	0.96	1.14	1.2	0.92
MV	0.58	0.6	0.58	0.64	1.38	0.8	0.76

Normal values for  $LT_{50}$  in 12 healthy male subjects on 6 separate samples.

\*  $LT_{50}$  : lag time to achieving 50% of maximal aggregation.

**TABLE AII.3 PLATELET AGGREGATION IN PRP IN RESPONSE TO COLLAGEN  
(19µg/ml) : NORMAL VALUES FOR LT<sub>50</sub>\* (mins)**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	1.54	1.6	1.72	2.5	1.42	2.46	1.87
TL	1.74	2.2	2.16	2.16	2.28	1.76	2.05
BS	1.6	1.36	1.36	1.4	1.54	2.28	1.59
MM	1.82	1.92	1.8	1.68	2.52	2.72	2.08
WB	1.94	2.46	1.72	1.86	1.6	2.48	2.01
MA	2.28	2.04	2.0	2.24	2.0	2.52	2.18
DM	2.48	2.24	2.24	2.3	1.86	2.36	2.25
NK	2.0	2.04	2.24	2.16	1.8	2.0	2.18
RR	1.62	1.92	2.24	2.06	2.52	2.2	2.09
RP	2.24	1.98	1.96	2.04	2.36	2.3	2.15
PL	2.2	1.88	1.85	1.62	2.02	1.6	1.86
MV	2.28	2.34	2.52	2.32	2.3	2.12	2.31

Normal values for LT<sub>50</sub> in 12 healthy male subjects on 6 separate samples.

**TABLE AII.4 PLATELET AGGREGATION IN PRP IN RESPONSE TO COLLAGEN  
(1.9µg/ml) : NORMAL VALUES FOR LT<sub>50</sub>\* (mins)**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	3.7	2.92	3.56	5.58	5.48	4.24	4.25
TL	5.18	5.68	5.18	5.44	5.4	4.2	5.18
BS	3.68	4.16	3.04	2.56	3.96	4.72	3.69
MM	3.16	5.44	4.68	5.52	5.12	4.78	4.78
WB	4.72	4.8	4.44	4.68	3.44	4.54	4.44
MA	5.54	5.2	5.56	4.96	4.68	5.48	5.24
DM	NA	NA	NA	NA	NA	NA	NA
NK	5.72	3.94	4.12	5.28	5.32	5.28	4.94
RR	4.64	5.02	5.34	5.28	3.92	5.30	4.92
RP	3.84	4.64	5.28	5.04	4.56	4.66	4.67
PL	3.88	3.9	3.28	3.16	4.44	2.96	3.74
MV	4.74	5.28	5.4	4.44	4.72	3.8	4.73

Normal values for LT<sub>50</sub> in 12 healthy male subjects on 6 separate samples.

\* LT<sub>50</sub> : lag time to achieving 50% of maximal aggregation.

NA : no aggregation.



**TABLE AII.5 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE (10 $\mu$ M) : NORMAL VALUES OF TMAX<sub>6</sub>\* (cm).**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	16.8	20.8	17.5	22.1	17.7	15.7	18.4
TL	19.3	17.0	16.5	15.0	14.4	17.2	16.6
BS	17.8	19.0	22.7	19.4	20.7	18.1	19.6
MM	19.1	15.3	17.5	18.2	17.6	17.5	17.5
WB	15.3	16.3	19.2	17.6	20.8	19.1	18.1
MA	16.0	15.8	16.9	17.1	16.3	16.6	16.5
DM	15.5	14.7	16.5	15.5	16.0	14.8	15.5
NK	13.6	16.7	17.1	17.7	18.1	16.8	16.7
RR	15.8	17.8	16.6	16.3	17.8	16.8	16.9
RP	16.6	15.5	16.5	19.3	14.5	14.4	16.1
PL	16.4	17.6	16.0	16.0	17.8	17.3	16.9
MV	18.2	17.9	14.4	19.0	22.3	19.7	18.6

Normal values for Tmax<sub>6</sub> in 12 healthy male subjects on 6 separate samples.

\* Tmax<sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist.

TABLE AII.6 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE  
(5 $\mu$ M) : NORMAL VALUES OF TMAX<sub>6</sub>\* (cm).

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	15.8	17.5	16.3	20.5	19.8	20.0	18.3
TL	19.0	17.9	16.8	15.4	16.4	16.5	17.0
BS	19.3	19.3	19.3	20.4	18.7	19.0	19.3
MM	18.1	14.5	18.3	16.4	15.7	16.6	16.6
WB	17.3	16.7	15.5	13.3	16.8	20.8	16.7
MA	16.5	15.6	18.2	16.2	14.3	17.2	16.3
DM	15.6	14.4	14.7	16.6	20.2	19.7	16.9
NK	12.9	16.1	15.5	16.3	16.5	19.3	16.1
RR	15.7	16.5	16.7	15.8	17.1	17.2	16.5
RP	16.2	17.8	15.0	16.6	16.3	15.4	16.2
PL	16.8	16.7	18.0	15.9	17.1	16.3	16.8
MV	15.2	15.4	17.0	18.2	16.9	16.5	16.5

Normal values for T<sub>max</sub><sub>6</sub> in 12 healthy male subjects on 6 separate samples.

\* T<sub>max</sub><sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist.

TABLE AII.7 PLATELET AGGREGATION IN PRP IN RESPONSE TO ADRENALINE (1 $\mu$ M) : NORMAL VALUES OF TMAX<sub>6</sub>\* (cm).

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	16.1	14.5	14.1	18.0	17.0	16.0	16.0
TL	6.0	14.4	15.4	5.0	10.6	9.1	10.1
BS	17.8	16.6	15.1	18.6	20.1	18.7	17.8
MM	16.4	13.8	17.3	16.8	17.5	15.9	16.3
WB	2.1	4.1	6.1	4.6	6.4	9.4	5.6
MA	16.3	14.7	16.6	16.1	16.8	14.8	15.9
DM	1.5	1.0	1.1	0.8	1.1	1.3	1.13
NK	16.6	15.3	17.4	16.9	17.5	16.2	16.6
RR	15.2	18.1	16.4	16.0	18.0	17.2	16.8
RP	15.4	14.3	16.1	14.5	15.5	13.8	14.9
PL	15.6	17.9	15.5	15.3	18.7	16.6	16.6
MV	18.3	17.3	18.1	18.1	21.7	15.7	18.2

Normal values for Tmax<sub>6</sub> in 12 healthy male subjects on 6 separate samples.

\* Tmax<sub>6</sub> : maximum height of aggregation trace 6 minutes after addition of agonist.

**TABLE AII.8 PLATELET AGGREGATION IN WHOLE BLOOD IN RESPONSE TO ARACHIDONIC ACID (0.66mM) : NORMAL VALUES FOR CHANGE IN IMPEDANCE ( $\Delta$  OHMS)**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	18.8	18.6	15.4	15.2	15.4	12.1	15.9
TL	17.4	19.2	19.5	13.5	12.4	9.5	15.3
BS	15.8	14.9	15.2	13.2	12.7	13.8	14.3
WB	12.9	12.8	7.6	5.6	9.5	4.2	8.8
PL	13.1	13.7	11.0	16.6	15.3	21.1	15.1
MV	11.4	23.3	19.5	16.0	14.4	12.9	16.3

Normal values for change in impedance from baseline levels in 6 healthy male subjects on 6 separate samples.

**TABLE AII.9 PLATELET AGGREGATION IN WHOLE BLOOD IN RESPONSE TO COLLAGEN (9.5 $\mu$ g/ml) : NORMAL VALUES FOR CHANGE IN IMPEDANCE ( $\Delta$ OHMS).**

Subjects	Sample Number						MEAN
	1	2	3	4	5	6	
WW	19.7	14.7	13.4	13.8	17.8	19.4	16.5
TL	16.3	11.0	15.2	12.3	10.9	12.0	13.0
BS	12.4	12.4	11.9	10.8	10.9	13.1	11.9
WB	6.1	4.1	8.5	5.0	9.8	8.7	7.0
PL	12.3	14.2	11.7	10.2	11.3	21.9	13.6
MV	19.0	19.6	8.0	10.2	12.6	15.3	14.1

Normal values for change in impedance from baseline levels in 6 healthy male subjects on 6 separate samples.

FIGURE AII.2 PLASMA CONCENTRATIONS OF B-THROMBOGLOBULIN AND PLATELET FACTOR 4:  
NORMAL VALUES IN HEALTHY SUBJECTS

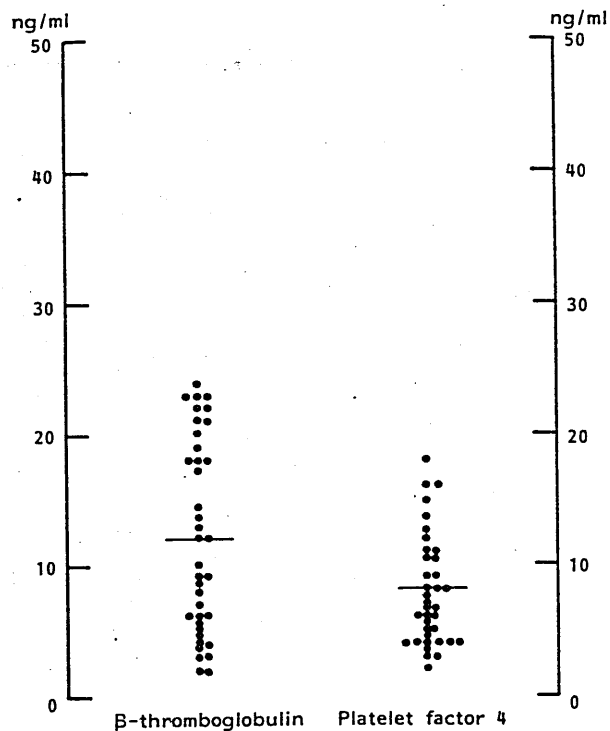


FIGURE AII.3 RELATIONSHIP BETWEEN PLASMA B-THROMBOGLOBULIN AND PLATELET FACTOR 4:  
EXCLUSION OF SAMPLES WITH ARTIFACTUALLY HIGH PLATELET FACTOR 4 LEVELS.

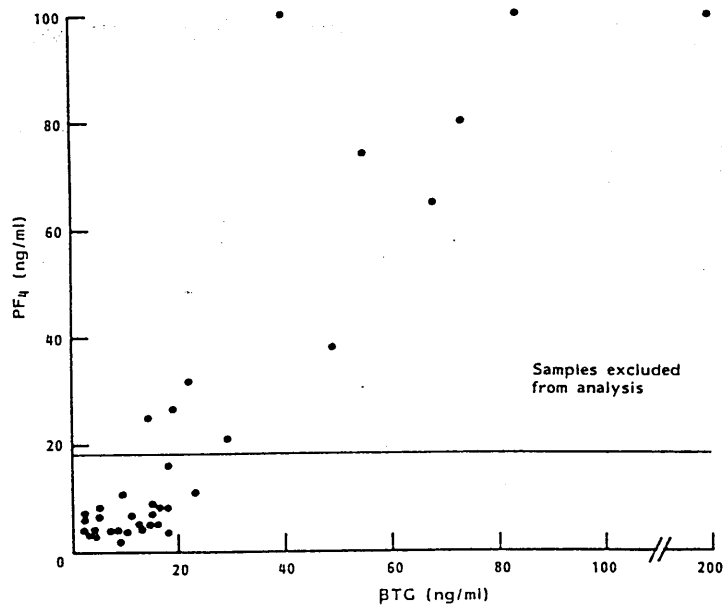
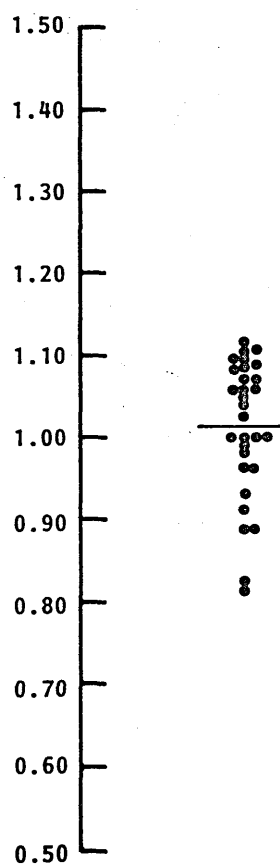


FIGURE AII.4 CIRCULATING PLATELET AGGREGATE RATIO: NORMAL VALUES IN HEALTHY SUBJECTS.



## APPENDIX III

### Reagents and Equipment



## Reagents

### 1. Reagents and materials for purification and derivatization of urinary eicosanoids:

Bonded phase phenylboronic acid columns  
(Analytichem International, Harbour City, CA.)  
C-18 Sep-Paks (Waters Associates, Milford, MA.)

Silicilic Acid plates (Whatman Chemical  
Separation Inc., Clifton, NJ.)

Pentafluorobenzyl bromide (Pierce Chemical  
Company, Rockford, IL.)

Diisopropylethylamine (Aldrich Chemical Company,  
Milwaukee, WI.)

Trifluoroacetamide (Supelco Inc., Bellefonte,  
PA)

Methoxyamine hydrochloride (Sigma Chemical  
Company, St. Louis, MO.)

Internal standards for Tx-M, thromboxane  
B<sub>2</sub> and PGI-M were kindly provided by Drs F.  
Fitzpatrick and J. Pike (Upjohn Company, Kalamazoo,  
MI)

All other chemicals (methanol, ethyl acetate  
etc) were obtained from Burdick and Jackson,  
Muskegon, MI.

2.Reagents and materials for platelet function studies:

Arachidonic acid (Sigma Chemical Company)

Collagen (Biodata Corporation, Horsham, PA.)

Adrenaline (Sigma Chemical Company)

Simplat Bleeding Time Device (General  
Diagnostics, Morris Plains, NJ.)

3.Reagents for radioimmunoassay of  
B-thromboglobulin, platelet factor 4 and thromboxane  
 $B_2$ :

Amersham B-thromboglobulin Kit (Amersham,  
Arlington Heights, IL.)

Platelet Factor 4 Radioimmunoassay Diagnostic  
Kit (Abbott Laboratories, North Chicago, IL.)

$PGE_1$  (Sigma Chemical Company)

Indomethacin (Sigma Chemical Company)

Rabbit anti-thromboxane  $B_2$  antibody  
(kindly provided by Dr RJ Workman, Vanderbilt  
University)

$I^{125}$ -thromboxane  $B_2$  (New England  
Nuclear, Boston, MA)

4.Drugs:

UK 38,485, aspirin 120mg and matching placebo

capsules (kindly provided by Dr P. Urquilla, Pfizer Inc., Groton, CT.)

CGS 13080 and matching placebo capsules (kindly provided by Dr N. Feliciano, Ciba-Geigy Corporation, Summit, NJ.)

Aspirin 1mg, 5mg, 20mg and 325mg capsules (prepared in Vanderbilt University Pharmacy)

Max-EPA (kindly provided by Dr D. Davies, Scherer Corporation, Troy, MI.)

Internal standards for aspirin and salicylate assays (kindly provided by Dr A.K. Pedersen, Vanderbilt University)

### Equipment

#### Gas chromatography/mass spectrometry:

Nermag 10-10C mass spectrometer (Nermag Corporation, Houston, TX.) linked to a Varian Vista 6000 gas chromatograph (Varian Corporation, Palo Alto, CA.)

Hewlett Packard 5980 (Hewlett Packard, Palo Alto, CA.)

#### Platelet Aggregometry:

Payton Dual-Channel Aggregometer (Payton

Associates, Buffalo, NY.)

Chrono-log Whole Blood Aggregometer (Chrono-log  
Corporation, Havertown, PA)

## APPENDIX IV

### Ethical Considerations

### Ethical Considerations

All the studies included in this thesis were approved by the local ethical committee, the Committee for the Protection of Human Subjects, of Vanderbilt University Medical Center, Nashville, Tennessee. When patients were attending the Veterans Administration Hospital, adjacent to Vanderbilt University, the studies were also approved by the ethical committee of that hospital. The aims and procedures of the study were explained to all the subjects individually by myself and by an experienced research nurse, Ms Cynthia Healy. All the participants signed a consent form prior to inclusion in the studies.

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## REFERENCES

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Inhibition of atherosclerosis by cod-liver oil in a  
hyperlipidemic swine model. New Engl J Med 1986;  
315:841-846.

### List of Publications

Parts of this thesis have already been published as outlined below:

- 1 Novel aspects of aspirin action.  
In : Bailey J M, ed. Prostaglandins, Leukotrienes and Lipoxins.  
Biochemistry, Mechanisms of Action, and Clinical Applications.  
New York : Plenum Press, 1985; 579-582.
- 2 The biochemical pharmacology of thromboxane synthase inhibition  
in man.  
Circulation 1985; 72 : 1194-1201.
- 3 Eicosenoid biosynthesis and platelet function with advancing  
age.  
Thrombosis Research 1986; 41 : 545-554.
- 4 Biosynthesis of thromboxane in patients with systemic sclerosis  
and Raynaud's phenomenon.  
Br Med J 1986; 292 : 1087-1089.
- 5 In vivo indexes of platelet and vascular function during fish-  
oil administration in patients with atherosclerosis.  
New Engl J Med 1986; 314 : 937-942.
- 6 Increased thromboxane biosynthesis in a human preparation of  
platelet activation : biochemical and functional consequences  
of selective inhibition of thromboxane synthase.  
Circulation 1986; 73 : 1300-1309.
- 7 Inhibition therapy of thromboxane formation in vivo and ex  
vivo : implications for therapy with platelet inhibitory drugs.  
Blood 1987; 69 : 180-186.

